ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 January 15; 13(1): 1-69

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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Monthly Volume 13 Number 1 January 15, 2022

World Journal of Diabetes

Monthly Volume 13 Number 1 January 15, 2022

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The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Manfredi Rizzo

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE
January 15, 2022

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https://www.wjgnet.com/bpg/gerinfo/242

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World Journal of Diabetes

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World J Diabetes 2022 January 15; 13(1): 1-4

DOI: 10.4239/wjd.v13.i1.1

ISSN 1948-9358 (online)

EDITORIAL

Acarbose is again on the stage

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Author contributions: Only Altay M contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Country/Territory of origin: Turkey

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

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Abstract

Acarbose is an agent that has been used to treat type 2 diabetes for about 30 years; it prevents postprandial hyperglycemia by inhibiting carbohydrate digestion in the small intestine. Since incretin-based treatments have been preferred over the last 10 to 15 years, the use of acarbose is not as common in treating type 2 diabetes as before. Some studies have shown that acarbose also produces a weight-loss effect by increasing glucagon-like peptide 1 (GLP-1). The positive effect of acarbose on GLP-1, and increasing evidence that it provides cardiovascular protection, suggests that acarbose may again be considered among the first-choice antidiabetic agents, as it was in the 1990s.

Key Words: Acarbose; Cardiovascular protection; Glucagon-like peptide 1; Obesity; Waist-to-height ratio

Core Tip: The prevention of obesity and reducing cardiovascular risks, together with blood glucose control in patients with type 2 diabetes, are the main components of the treatment's goals. New studies show that acarbose can provide the expected benefits of an ideal antidiabetic drug by increasing both insulin sensitivity and glucagon-like peptide 1 levels.

Citation: Altay M. Acarbose is again on the stage. *World J Diabetes* 2022; 13(1): 1-4
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/1.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.1

Received: March 31, 2021
Peer-review started: March 31, 2021
First decision: June 5, 2021
Revised: June 19, 2021
Accepted: December 22, 1021
Article in press: December 22, 1021
Published online: January 15, 2022

P-Reviewer: Cigrovski Berkovic M, Forlano R, Sun XD, Xu R
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

INTRODUCTION

Obesity is a key factor in the prevalence of type 2 diabetes mellitus (T2DM) worldwide. Therefore, in treating diabetes, researchers focus on the consequences of eliminating the negative effects of obesity, especially abdominal obesity, on reducing cardiovascular events and death. In a recently published study, Song *et al*[1] aimed to examine the effect of acarbose on abdominal obesity, and its determining factors in comparison with metformin[1]. They evaluated Metformin and AcaRbose in Chinese as the initial Hypoglycemic treatment (MARCH) study data[2] using a new anthropometric measure: Waist-to-height ratio (WHtR). The MARCH study is a randomized, open-labeled, noninferiority trial on Type 2 diabetes patients that was published in 2014[2]. It has been showen in this study that acarbose treatment is as effective and safe as metformin at the 24th and 48th weeks. A group of 343 patients who were newly diagnosed with T2DM were treated with acarbose, and 333 other patients were treated with metformin. The new report by Song *et al*[1] clarified that WHtR had significantly decreased in both groups in the 24th week after treatment, with women showing a more pronounced decrease. Between the beginning of the study and the 24th week of the treatment, the change in the waist-to-height ratio (ΔWHtR) was divided into two sets with large differences in one group and small differences in the other, thus, these data were subject to post-hoc analysis. In the acarbose group, women and those with a lower area under the glucagon-like peptide 1 (GLP-1) curve (AUCGLP-1) had a greater ΔWHtR. Among those using metformin, weight loss was greater in women as well as those with a high baseline AUCGLP-1. In conclusion, Song *et al*[1] found a relationship between high WHtR in the treatment of acarbose with gender, GLP-1 level, fasting glucose, and lipid profile. In addition, Song *et al*[1] emphasized the importance of WHtR for the measurement of abdominal obesity. They argued that, in both groups, a greater reduction in waist circumference in women was independent of the drug and was due to women's excessive desire and attempts to lose weight. The study observed that the circulating GLP-1 level increased over time in acarbose users. Previous studies reported that alpha glucosidase enzyme inhibition increased circulating GLP-1 levels by stimulating GLP-1 secretion and inhibiting dipeptidyl peptidase 4 (DPP-4) enzymes in healthy and T2DM patients[3-7]. Moreover, a recently published study showed this effect to be inhibited by exendin, a GLP-1 receptor antagonist[8]. This study found that acarbose is more effective for abdominal obesity, especially in those with low GLP-1 levels. The effect of lifestyle change on the results was not evaluated in the article, which is an important limiting factor.

The work of Song *et al*[1] throws up a question: "What role should acarbose play in the treatment of diabetes?" While acarbose continued to be part of diabetes guidelines and treatment algorithms, the appearance of new treatment agents in the last 10 to 15 years pushed acarbose to the background. In fact, there are large-scale studies that solidify the role of acarbose in treating impaired glucose tolerance (IGT) and T2DM. Over the past year, however, acarbose seems to have regained its importance. Prominent studies, such as the Study to Prevent Non-Insulin-Dependent Diabetes Mellitus (STOP-NIDDM) and the Acarbose Cardiovascular Evaluation (ACE) study, show that acarbose prevents the development of diabetes regardless of age, gender, and body mass index[9,10]. It has also been found that acarbose reduces cardiovascular events in patients with IGT and T2DM. In a recently published study, Zhang *et al*[11] found a 50% relative risk (RR) reduction in myocardial infarction and a 52% RR reduction in all-cause deaths after a 10-year follow-up with regard to acarbose therapy in patients with T2DM[11]. This effect is due to the reduction of oxidative stress caused by the lowering of postprandial two-hour blood sugar. Some studies have claimed that it is effective in quickly providing joint target controls. However, the fact that the study was conducted only in Chinese patients is an important limiting factor. An increasing number of studies focus on the mechanisms with which acarbose acts in diabetes treatment and how it provides additional benefits[8]. The possible effect mechanisms of acarbose on diabetic patients are shown in Table 1.

Acarbose inhibits carbohydrate digestion by competitively inhibiting the alpha glucosidase enzyme in the small intestine lumen. Consequently, it reduces glucose absorption, prevents postprandial hyperglycemia and hyperinsulinemia, and increases insulin sensitivity[12]. For this reason, it has been used in clinical practice since the 1990s, whether in monotherapy for mild cases of type 2 diabetes or as a combination agent with insulin and other antidiabetics in severe and advanced cases. Some studies have shown that acarbose has positive effects on intestinal flora[13]. In order to reduce gastrointestinal intolerance, a daily dose of 50 mg is offered just before meals, and a dose of 100 mg is offered three times a day after four to six weeks, when weekly titrations are reached. Acarbose can decrease hemoglobin A1c (HBA1c) by 0.5% to

Table 1 The possible mechanisms of effects of acarbose on diabetic patients

Type of effect	Net effect	Mechanism
Glucose absorption	Decrease	Competitively inhibits α-glucosidases absorption in small intestine
Insulin sensitivity	Increase	Lowers the postprandial blood glucose and insulin levels
DPP-4 activity	Decrease	Increases postprandial glucose in small intestine
Circulating GLP-1 level	Increase	Stimulates GLP-1 secretion in small intestine
Intestinal content	Increase	Positively effects microbiota *via* increasing content of oligosaccharides in the digestive tract

GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase 4.

1.5% and is especially effective on postprandial hyperglycemia[12].

The following are the advantages of acarbose: It is one of the rare agents that has been shown to prevent diabetes in the pre-diabetic period; the rate of hypoglycemia is low; its annual cost is lower than that of new antidiabetic drugs; it has weight-loss properties, or at least is weight neutral; it has a positive effect on the lipid profile by lowering the triglyceride level; and there is increasing evidence to show that it reduces the risk factors of cardiovascular disease. However, it shouldn't be forgotten that this hasn't yet been proven in Cardio Vascular Outcome Trials (CVOTs).

The disadvantages of acarbose are that it has to be used three times a day, and gastrointestinal side effects, such as gas, bloating, and diarrhea are relatively frequent.

CONCLUSION

In my opinion, we should remember that acarbose is an effective alternative to controlling postprandial hypoglycemia in countries that predominantly consume carbohydrates, like China or Turkey. The increasing evidence on its effects on GLP-1 and cardiovascular protection may lead to an extension of its use. It seems that acarbose, which has a high efficacy and is safe in terms of its side-effect profile, will be at the forefront of diabetes guidelines in the near future.

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World Journal of Diabetes

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World J Diabetes 2022 January 15; 13(1): 5-26

DOI: 10.4239/wjd.v13.i1.5

ISSN 1948-9358 (online)

REVIEW

Polycystic ovary syndrome and type 2 diabetes mellitus: A state-of-the-art review

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Author contributions: All authors equally contributed to this manuscript.

Conflict-of-interest statement: The authors have no conflict of interest to disclose.

Country/Territory of origin: Greece

Specialty type: Endocrinology and Metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Polycystic ovary syndrome (PCOS) often coexists with a wide spectrum of dysglycemic conditions, ranging from impaired glucose tolerance to type 2 diabetes mellitus (T2D), which occur to a greater extent compared to healthy body mass index-matched women. This concurrence of disorders is mainly attributed to common pathogenetic pathways linking the two entities, such as insulin resistance. However, due to methodological flaws in the available studies and the multifaceted nature of the syndrome, there has been substantial controversy as to the exact association between T2D and PCOS which has not yet been elucidated. The aim of this review is to present the best available evidence regarding the epidemiology of dysglycemia in PCOS, the unique pathophysiological mechanisms underlying the progression of dysglycemia, the most appropriate methods for assessing glycemic status and the risk factors for T2D development in this population, as well as T2D risk after transition to menopause. Proposals for application of a holistic approach to enable optimal management of T2D risk in PCOS are also provided. Specifically, adoption of a healthy lifestyle with adherence to improved dietary patterns, such the Mediterranean diet, avoidance of consumption of endocrine-disrupting foods and beverages, regular exercise, and the effect of certain medications, such as metformin and glucagon-like peptide 1 receptor agonists, are discussed. Furthermore, the maintenance of a healthy weight is highlighted as a key factor in achievement of a significant reduction of T2D risk in women with PCOS.

Received: April 20, 2021
Peer-review started: April 20, 2021
First decision: June 5, 2021
Revised: June 30, 2021
Accepted: December 25, 2021
Article in press: December 25, 2021
Published online: January 15, 2022

P-Reviewer: Wu QN
S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

Key Words: Polycystic ovary syndrome; Diabetes; Insulin resistance; Dysglycemia; Women

Core Tip: Polycystic ovary syndrome (PCOS) often coexists with a wide spectrum of dysglycemic conditions, ranging from impaired glucose tolerance to type 2 diabetes mellitus (T2D), which occur to a greater extent compared to healthy body mass index-matched women. This review provides the most current knowledge on the different aspects of T2D in women with PCOS, including epidemiology, common pathophysiologic mechanisms, and methodology employed for dysglycemia assessment, as well as to scrutinize the risk factors for T2D development and to suggests the optimal management of these women in the context of T2D risk reduction.

Citation: Livadas S, Anagnostis P, Bosdou JK, Bantouna D, Paparodis R. Polycystic ovary syndrome and type 2 diabetes mellitus: A state-of-the-art review. *World J Diabetes* 2022; 13(1): 5-26
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/5.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.5

INTRODUCTION

Polycystic ovary syndrome (PCOS) constitutes the most common endocrine disorder in women of reproductive age, affecting 6%-15% of the of the global population[1]. PCOS is a multifaceted, ever-changing disease and a challenging disorder for the caring physician due to the continuous need for treatment modifications and adjustments based on the patient's fluctuating needs and preferences throughout the course of her lifetime. Apart from oligo- or amenorrhea and/or clinical or biochemical hyperandrogenism, impaired glucose homeostasis has also been observed in patients with PCOS[1,2]. In particular, evidence from large prospective cohorts has shown progression to either prediabetes or type 2 diabetes mellitus (T2D) over time[3,4]. The emergence of T2D in PCOS can be anticipated to some extent given that the two prerequisites for T2D development, insulin resistance (IR) and ß-cell dysfunction, are frequently present in women with PCOS. Indeed, IR, which is a key player in underlying PCOS pathophysiology, has been documented in the vast majority of women suffering from the syndrome in comparison to their healthy body mass index (BMI)-matched peers. An additive effect of obesity on the degree of IR reported in these women should also be taken into account[5]. Meanwhile, the prevalence of pancreatic ß-cell dysfunction is much higher in these patients compared to their regularly ovulating, non-hyperandrogenic peers[6].

Nevertheless, there is an ongoing debate as to whether PCOS itself constitutes a risk factor for T2D or whether T2D predominantly occurs in the context of obesity in affected patients[7-9]. A recent meta-analysis of genetic studies suggests that there is no inherent T2D risk in PCOS and that T2D instead occurs as a result of either increased adiposity or hyperandrogenemia[10]. On the other hand, PCOS constitutes a polygenic trait and elegant studies have shown that clusters of genes leading to metabolic disturbances are different from those associated with overt hyperandrogenic signs in PCOS women[11]. Therefore, a genetic component of dysglycemia among PCOS women should be considered.

The presence of altered glycemic status, although a universal finding, is challenging to the clinician for several reasons. One is that the reported incidence of dysglycemia, which includes impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and T2D, varies among studies. Furthermore, agreement over a definitive recommendation regarding the optimal method for assessment of glycemic status has not been reached to date.

The aim of this narrative review was to provide the most current knowledge on the different aspects of T2D in women with PCOS, including epidemiology, common pathophysiologic mechanisms, and methodology employed for dysglycemia assessment, as well as to scrutinize the risk factors for T2D development and to

suggest the optimal management of these women in the context of T2D risk reduction.

EPIDEMIOLOGY OF DYSGLYCEMIA IN PCOS

In general, the prevalence of dysglycemia is significantly higher in women with PCOS compared to their healthy BMI-matched peers. With regard to T2D, in normal women of reproductive age, the mean prevalence of T2D is 1%-3%[12], whereas in PCOS, its prevalence ranges from 1.5 to 12.4%, with a median value of 4.5%. This wide range partly depends on the age of the studied subjects, with the higher incidence (12.4%) recorded in a study evaluating mostly perimenopausal women with PCOS and a mean age of 46 years[13]. In the remaining studies, the mean age of the studied population ranged from 25 to 30 years. Another factor closely associated with T2D prevalence is ethnic variation, since a prevalence of 6.3% and 10.1% has been reported in two studies from Asia[14,15], reflecting the rising prevalence of T2D in Asia[16], a trend that has recently been corroborated in a large meta-analysis[17]. Finally, one more factor pertains to the criteria applied for PCOS diagnosis. For example, a higher degree of dysglycemia is anticipated in women diagnosed with the 1991 National Institutes of Health (NIH) criteria in comparison with the mild phenotype D of those diagnosed with the 2003 Rotterdam criteria, this due to the lower degree of IR observed in the latter group[18]. On the other hand, this logical assumption was not confirmed by a recent study evaluating more than 2000 women, which showed that T2D prevalence was similar among patients with different PCOS phenotypes[19].

Conflicting data exist regarding the prevalence of intermediate hyperglycemia, namely, IGT and IFG. The prevalence of IGT in PCOS ranges from 4%-35.4%, with an average of 16.6%; in contrast, the corresponding prevalence in the healthy peers of women with PCOS ranges from 4%-8%[12]. The reasons for this very high heterogeneity have not been fully elucidated; however, ethnic susceptibility, the various criteria applied for PCOS diagnosis, as well as age and BMI distribution in the different studied groups could partly explain this diversity. Likewise, IFG prevalence as reported in the literature ranges from 2%-21%, the average being 10.8%, higher than that of the non-PCOS population, in which it is approximately 5.9% (range 4%-8.7%)[20]. In addition to the reasons provided above, the diagnostic criteria employed to diagnose IFG also play an important role, with IFG cut-offs differing significantly between the American Diabetes Association (ADA) and the World Health Organization (WHO) clinical practice guidelines. Therefore, it is not surprising that the prevalence of IFG was higher in studies using the stricter ADA criteria[21] than in those using the corresponding WHO cut-off values[22]. The prevalence of dysglycemia as reported in different studies according to the country where the study was performed, the subjects' age and BMI, and the diagnostic criteria to confirm the diagnosis of either PCOS or glycemia are presented in Table 1.

PATHOPHYSIOLOGY LINKING PCOS WITH INCREASED RISK OF T2D

PCOS pathophysiology is characterized by a combination of androgen excess and ovulatory dysfunction. Although numerous studies have endeavored to identify the underlying pathogenetic mechanisms, this particular 'Holy Grail' of endocrinology has not as yet been uncovered. Irrespective of the theoretical perspective, it is widely accepted that the unusually variable phenotype in affected patients is produced by the combined effects of two separate, yet deeply intertwined, mechanisms, which are androgen over-activity (elevated androgen concentrations or hyperandrogenism) and IR[23].

IR represents a state of disrupted insulin binding to its receptor or ineffective activation of the latter by insulin, thereby forcing the pancreatic β-cells to release large amounts of insulin into the circulation in order to maintain euglycemia[24]. Such a state of chronic pancreatic stress leads to impaired glucose homeostasis, initially manifesting as IFG or IGT; however, once large numbers of islet β-cells have succumbed to stress, it leads to T2D. IR and glucose homeostasis abnormalities have been described in up to 70% of women with PCOS[25]. As early as 1980, eight obese subjects with PCOS were found to have higher serum glucose and insulin concentrations, in both a fasting state and after stimulation by an oral glucose load, compared with six obese unaffected women, despite the latter being statistically significantly more obese[26]. Even though obesity is a key risk factor for IR and T2D development in the general population, women with PCOS have higher insulin concentrations in

Table 1 Incidence of dysglycemia in women with polycystic ovary syndrome

Ref.	Sample size	Country	PCOS criteria	T2D criteria	Age (yr)	BMI (kg/m²)	IFG (%)	IGT (%)	T2D (%)
Rajkhowa *et al*[142], 1996	90	UK	NIH	WHO	26 (15-39)	31.6 (18-48)	?	9	2
Legro *et al*[61], 1999	254	USA	NIH	WHO	14-44	32 ± 3	?	31	7.5
Ehrmann *et al*[62], 1999	122	USA	NIH	ADA	25 ± 0.7 (13-40)	30-43	9	35	10
Gambineri *et al*[3], 2004	121	Italy	Rotterdam	WHO	14-37	20-38	?	15.7	2.5
Legro *et al*[143], 2005	71	USA	NIH	ADA	30 ± 6	29 ± 6.4	?	25	10
Chen *et al*[144], 2006	102	China	Rotterdam	WHO	24.2 ± 6	21.7 ± 4	?	20.5	1.9
Mohlig *et al*[64], 2006	264	Germany	NIH	WHO	28 ± 0.4	30 ± 0.4	?	14.3	1.5
Vrbikova *et al*[145], 2007	244	Czech Republic	Rotterdam	ADA	27 ± 7.5	27 ± 6.9	12.3	9.4	1.6
Gagnon *et al*[146], 2007	105	Canada	NIH	ADA	28.3 (14-47)	35.5 (19-54)	?	23	5
Dabadghao *et al*[63], 2007	372	Australia	Rotterdam	ADA	30 ± 5 (15-62)	35 ± 8	3	15.6	4
Espinos-Gomez *et al*[147], 2008	102	Spain	NIH	WHO	26 ± 6	30.2 ± 8	?	10.7	7.7
Cheung *et al*[148], 2008	295	China	Rotterdam	ADA	30 ± 6	25 ± 5.9	9.2	10.5	7.5
Bhattacharya *et al*[149], 2009	264	India	Rotterdam	WHO	24 ± 4	27 ± 4.5	?	14.4	
Seneviratne *et al*[15], 2009	168	Sri Lanka	Rotterdam	WHO	29 ± 4 (20–40)	25.92 (16-39)	?	23.2	10.1
Lee *et al*[50], 2009	194	Korea	Rotterdam	ADA	27 ± 5	24 ± 4	17		1
Wei *et al*[51], 2009	356	China	Rotterdam	WHO	32 ± 4 (19-44)	22 ± 4.2	?	7.6	**3,1**
Zhao *et al*[150], 2010	818	China	Rotterdam	ADA	25 ± 5	?	8.5	35.4	**4**
Stovall *et al*[151], 2011	78	USA	NIH	ADA	26 ± 6.4	29 ± 6 (18-43)	2	14	**?**
Celik *et al*[66], 2013	252	Turkey	Rotterdam	ADA	24 ± 5	26 ± 5.7	?	14.3	**2**
Veltman-Verhulst *et al*[21], 2013	226	Netherlands	Rotterdam	ADA	29.6 ± 4	27 ± 6.7	21	4	**3.5**
Lerchbaum *et al*[152], 2014	714	Austria	Rotterdam	ADA	27 (23-32)	24.2 (21-30)	12.8		1.5
Vrbikova *et al*[145], 2014	330	Czech Republic	Rotterdam	ADA	27.8 ± 7	27.6 ± 6	12	8.8	**3**
Amato *et al*[22], 2015	241	Italy	Rotterdam	WHO	24 ± 6 (14-43)	30 ± 6 (18-50)	11.6	5.4	**1.7**
Ganie *et al*[14], 2015	2014	India	Rotterdam	ADA	23 ± 5.4	25 ± 4.4	14.5	5.9	**6.3**
Gracelyn *et al*[153], 2015	200	India	Rotterdam	ADA	16-40	?	?	14.5	**1.5**
Li *et al*[154], 2016	2436	China	Rotterdam	ADA	27	21.56	13.5	19.8	**3.9**
Ollila *et al*[127], 2017	265	Finland	Rotterdam	WHO	46	28.6 ± 6	?	?	**12.4**
Pelanis *et al*[13], 2017	876	Sweden	Rotterdam	ADA	29 (25-34)	28 (23-33)	11	12	**3**
Zhang *et al*[19], 2018	378	China	Rotterdam	IDF	27 ± 4.4	30 ± 4.3	31.5		8.7
Ortiz-Flores *et al*[155], 2019	400	Spain	Rotterdam	WHO	26 (14-49)	28.6 (22-34)	14	14.5	2.5

NIH: National Institutes of Health; T2D: Type 2 diabetes mellitus; PCOS: Polycystic ovary syndrome; ADA: American Diabetes Association; WHO: World Health Organization.

response to an oral glucose load as compared to unaffected subjects, even in the absence of obesity[27,28]. The only clinical sign of IR is acanthosis nigricans, which correlates well with IR in either obese or lean affected individuals[29].

Of course, women with PCOS are equally exposed to the well-established association of obesity and higher degree of IR as those without PCOS. Indeed, a study comparing 198 obese and 201 non-obese women with PCOS (obesity definition: BMI > 27 Kg/m²) found that obesity was associated with lower insulin sensitivity when a

variety of oral glucose tolerance test (OGTT)-derived indices was used[30]. In contrast to the latter findings, a study employing the impractical gold standard method to assess IR, namely, the hyperinsulinemic euglycemic clamp, it found more pronounced insulin secretion in lean women with PCOS compared to controls, though without significant differences in insulin sensitivity, while it confirmed the presence of higher IR compared to controls only in obese women with PCOS[31].

In addition, women with PCOS present with enhanced luteinizing hormone pulsatility, producing increased secretion of ovarian and adrenal androgens, which, along with IR, are key features of the syndrome. A meta-analysis of cross-sectional studies including 4795 women from the general population found higher testosterone concentrations in patients with T2D compared to controls[32]. In addition bioavailable testosterone correlated significantly with IR, with higher concentrations predicting the development of T2D[33]. Similar results were obtained from a systematic review and meta-analysis pooling data from the Rotterdam Study and other previously published studies, which found that subjects at the highest tertile of free androgen index had a 42% higher risk of developing T2D, in a complex multivariate analysis controlling, among others, for age, BMI, glucose, and insulin concentrations[34].

Given that PCOS is characterized by markedly higher androgen concentrations compared to those in the unaffected population, an association between the syndrome and T2D constitutes a rational hypothesis. In fact, such correlations were originally reported almost 40 years ago[35]. Ever since, multiple studies have confirmed this relationship in both lean and obese women with PCOS. A significant positive association between testosterone concentrations and IR has been described in lean women with PCOS using OGTT-derived indices[3,36] and measures of glucose disposition in hyperinsulinemic euglycemic clamps[37]. Even though PCOS is mainly characterized by hyperandrogenemia of ovarian origin, a subgroup of patients exhibits adrenal androgen hypersecretion, with the most important being dehydroepiandrosterone sulphate. In the latter subgroup, hyperandrogenemia does not seem to correlate with IR indices or metabolic abnormalities in most[38-42], but not all, studies[43,44]. It is a riddle that remains as yet unresolved, especially taking into consideration that such an association does seem to exist in other conditions of adrenal hyperandrogenism, such as premature adrenarche/pubarche[45].

Despite the similar pathophysiology of PCOS and glucose homeostasis abnormalities, the differences between lean and obese women with PCOS are remarkable. This was shown by a recent study in which lean women with PCOS failed to improve their whole body insulin action, measured using a hyperinsulinemic euglycemic clamp after 14 wk of controlled and supervised exercise training, in contrast to controls[46]. Therefore, it came as no surprise when a recent meta-analysis of 13 studies including almost 279000 subjects identified a markedly elevated risk for T2D among women with PCOS compared to unaffected women [5.9% *vs* 2.0%; relative risk (RR): 3.00, 95%CI: 2.56-3.51; I^2 = 83%][47]. Similar results were found *via* a meta-analysis recently presented by our group: in this systematic review, 23 studies were lumped together, incorporating data from 319870 participants who comprised 60337 patients with PCOS and 8847 cases with T2D (RR: 3.45, 95%CI: 2.95-4.05). In our study, the effect of BMI on the risk of T2D was assessed, pooling data from three studies, which identified a pronounced effect of obesity. In particular, the RR for developing T2DM in overweight/obese and non-obese women with PCOS, as compared to their non-PCOS counterparts, was 5.75 (95%CI: 1.20-27.42) and 3.34 (95%CI: 0.03-400.52), respectively. Moreover, the RR for developing T2D in overweight/obese compared to lean women with PCOS was 3.96 (95%CI: 1.22-12.83)[48].

Meanwhile, the role of aging in T2D development should certainly not be underestimated. This has been demonstrated in, inter alia, a subgroup analysis of 345 Dutch women with PCOS, who were part of a large cohort study evaluating aging in women with PCOS (APOS study)[49], where the interaction of age and BMI was the most significant variable in predicting T2D in logistic regression analysis. Moreover, data assembled from several studies have also pointed to a positive association of age and BMI with T2D or intermediate hyperglycemia among women with PCOS[19,50,51]. On the other hand, a cross-sectional study conducted by our group found that aging might exert a protective effect in women with PCOS with regard to IR. In particular, obese women with PCOS demonstrated the same degree of IR through the years, although this was not the case for their lean peers in whom a gradual improvement was observed with aging (Figure 1)[52]. Furthermore, a large cross-sectional study (n = 763 normal-weight women with PCOS, according to the Rotterdam criteria; 376 controls) exhibited a parallel decrease of homeostasis insulin resistance assessment (HOMA-IR) index with free androgen index, suggesting a potential mechanism regulating this process (Figure 2)[53]. Specifically, the gradual reduction of androgen

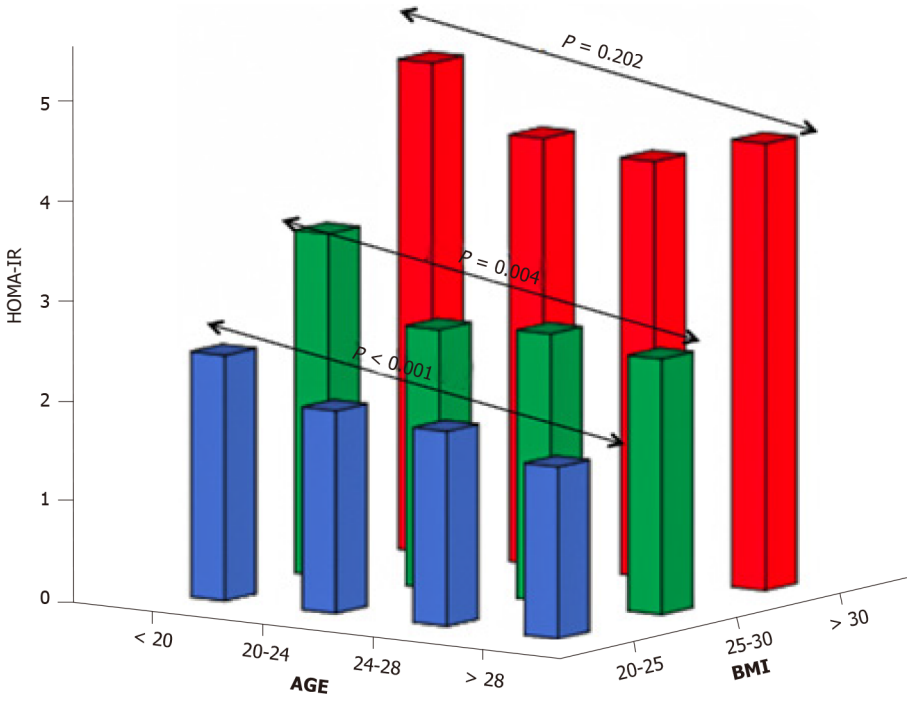

Figure 1 The gradual improvement of insulin resistance over the years in normal weight (blue bars) and overweight (green bars) women with polycystic ovary syndrome, but not in their obese counterparts (red bars). Adapted from[52]. HOMA-IR: Homeostatic model assessment for insulin resistance; AGE: Advanced glycation end-product; BMI: Body mass index.

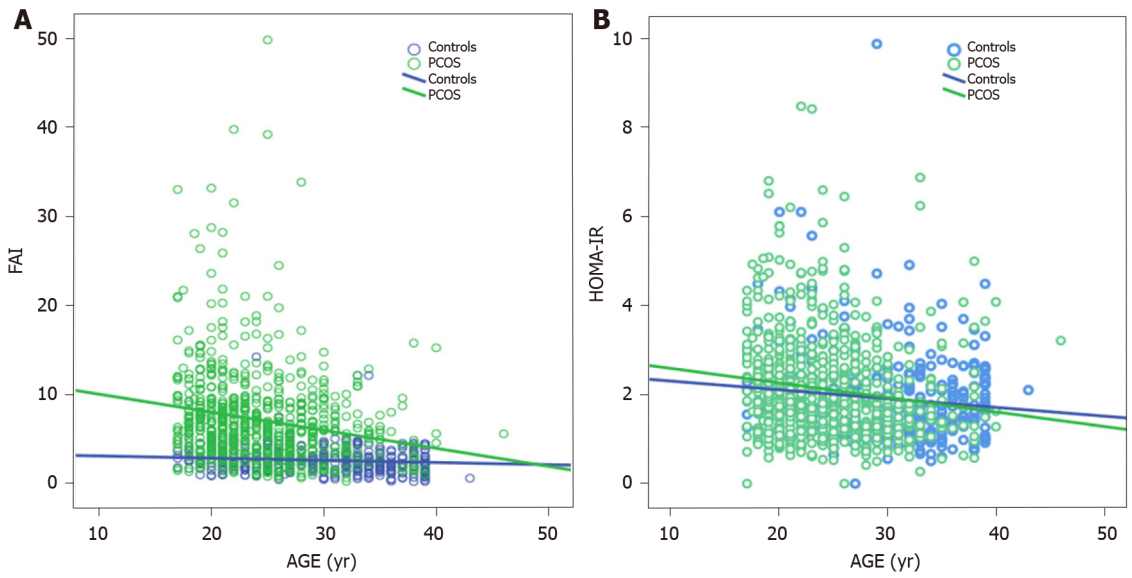

Figure 2 The gradual decrease of homeostatic model assessment for insulin resistance and free androgen index in normal weight women with polycystic ovary syndrome, compared with controls. Adapted from[53]. A: Free androgen index; B: Homeostatic model assessment for insulin resistance. PCOS: Polycystic ovary syndrome; FAI: Free androgen index; HOMA-IR: Homeostatic model assessment for insulin resistance.

production observed in women with PCOS after their third decade of life partly explains the absence of deterioration of IR through the years which is common in the general population.

ASSESSMENT OF DYSGLYCEMIA IN PCOS

According to the Wilson and Jungner criteria, screening for a disease is essential when that condition constitutes an important health problem, an accepted treatment for patients with recognized disease exists, and facilities for diagnosis and treatment are

available. Furthermore, there should be an identifiable latent or early symptomatic stage, and the natural history of the condition, including development from latent to clinical disease, must be adequately understood[54]. Furthermore, there should be an agreement upon policy as to whom to treat, taking into consideration the patients' resources. The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditures for medical care as a whole, while case finding should be a continuing process and not a "one-off" strategy. Finally, there should be an effective screening test or examination and that test should be acceptable to the population[54]. It is obvious that PCOS covers all the prerequisites described above and, therefore, in all consensus statements by several experts, screening for T2D is recommended in women with PCOS (Table 2).

However, whether glycemic status should be evaluated in every woman suffering from PCOS or in certain subgroups, as well as which is the best method for this assessment, are to date unanswered questions. With regard to which patients should be screened, there are at present two points of view. One, supported by the Endocrine Society, the Androgen Excess and Polycystic Ovary Syndrome Society, as well as by the guidelines on PCOS diagnosis and management developed in Australia, suggests universal screening in all women with PCOS[55-57]. On the other hand, a number of experts recommend screening in women with at least one risk factor, such as age >40 years, family history of either T2D or gestational diabetes mellitus, and/or obesity[58-60]. However, the latter recommendations have not been supported by solid data, since studies arguing either in favor of or against them have been published[4,61]. For example, the family history of T2D criterion has strong supportive data in studies from the USA and Australia[62,63], but not in studies originating from Italy and Germany[9,64]. Accordingly, these criteria, although reasonable, appear ultimately to be arbitrary and would not reflect the different nature of T2D development in PCOS compared to that in the unaffected population. In a similar manner, most of the studies in favor of these recommendations did not evaluate in detail the impact of age, obesity, and hyperandrogenemia on the development of dysglycemia and, thus, seem to increase controversy over this matter[4,65].

There is disagreement among experts as to whether fasting plasma glucose (FPG), OGTT, or glycated hemoglobin (HbA1c) is the best laboratory method to assess glycemic status in a patient with PCOS, despite robust data strongly pointing to OGTT as being the most accurate[66,67]. The main arguments against OGTT use are that the modality is more complex, expensive, and time-consuming than the other two screening methods. Moreover, it is characterized by high variability and its results are dependent on height[68]. However, OGTT is considered the gold standard for T2D diagnosis because it constitutes a standardized test that is easily performed and is the only method able to detect IGT, of utmost importance for women with PCOS[69]. Indeed, given that the risk of T2D development in women with IGT is considerably higher than in those with normal glucose tolerance or those with IFG[9], this at-risk population can greatly benefit from early lifestyle modification and/or pharmacological intervention[70].

In addition, the ADA and WHO are in agreement regarding the glucose concentration cut-off for the diagnosis of IGT, which is not the case for either FPG or HbA1c values. Furthermore, in several studies it has been shown that a single measurement of FPG could misclassify a substantial number of patients with either IGT or T2D, ranging from 20%-40%, as having normal glucose homeostasis[21,64,71]. This figure is certainly not negligible given that women with PCOS are at risk for T2D, even from their early reproductive years, compared to their healthy peers. Other benefits of an OGTT are that it can be applied in patients with iron deficiency, a condition commonly encountered in women of reproductive age, while the parallel measurement of insulin concentrations after a glycemic load provides the clinician with an accurate estimate of the degree of existing IR[72].

After the ADA's recommendation for a single HbA1c measurement as an accurate index for T2D diagnosis, its use has been advocated by several research groups and international guidelines (Table 2). HbA1c cannot, however, be used for the diagnosis of dysglycemia in women suffering from PCOS for a number of reasons. First, in this group of patients, periods of oligomenorrhea are followed by periods of heavy bleeding or sychnomenorrhea and this menstrual pattern could result in major changes in hematocrit and/or ferritin levels. Since HbA1c is dependent on these parameters, iron depletion and loading might lead to significant variations in HbA1c concentrations over time, independently of the patient's glycemic status[73]. Moreover, the specificity of HbA1c in the diagnosis of dysglycemia has been questioned in overweight and obese subjects[74], who constitute the largest group in the PCOS population. Additionally, the cut-off point for HbA1c is mainly based on the

Table 2 Guidelines regarding oral glucose tolerance test upon diagnosis of Polycystic ovary syndrome

Ref.	OGTT recommended upon diagnosis in all women with PCOS	Follow-up with OGTT
Joint AACE/ACE and AE-PCOS society[56]	Yes	(1) Yearly in women with IGT; and (2) Every 1–2 years based on BMI (not specified) and family history of T2D
Australian NHMRC[57]	Recommended if one or more criteria exist: (1) BMI > 25 kg/m^2 or in Asians > 23 kg/m^2; (2) History of IFG, IGT, GDM; (3) Family history of T2D; (4) Arterial hypertension; and (5) High-risk ethnicity	Every 1-3 years, based on presence of other diabetesrisk factors
Endocrine Society[55]	Yes	Every 3–5 years (Sooner if additional risk factors for T2D develop)
Royal College of Obstetricians andGynecology[59]	Recommended if one or more criteria exist: (1) BMI ≥ 25 kg/m^2; (2) Age ≥ 40 years; (3) Previous GDM; and (4) Family history of T2D	Yearly in women with IGT or IFG
AE-PCOS Society[58]	Recommended if one or more criteria exist: (1) BMI ≥ 30 kg/m^2; (2) Age ≥ 40 years; (3) Previous GDM; and (4) Family history of T2D	Every 2 years in women with risk factors (Sooner if additional risk factors for T2D develop)
ESHRE and ASRM[60]	Recommended if BMI ≥ 27 kg/m^2	Not specified

OGTT: Oral glucose tolerance test; PCOS: Polycystic ovary syndrome; T2D: type 2 diabetes mellitus; BMI: Body mass index; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance.

established association between HbA1c and microvascular disease in patients with established T2D. However, women with PCOS are younger and healthier overall when initially diagnosed with the syndrome, while dysglycemia does not always lead to T2D in this population compared to those evaluated in the original study of HbA1C validation[75], further calling HbA1c application into question.

Besides this, HbA1c is a costly procedure, harmonization of HbA1c assays around the globe has not yet been carried out effectively, significant variation across ethnicities has been described, and international standardization is not as yet complete [76]. The diagnostic performance of HbA1c as a marker of glucose intolerance is further compromised by the discordance between the diagnostic criteria for prediabetes proposed by the WHO [42 mmol/mol (6.0%)] and those by the ADA [39 mmol/mol (5.7%)], producing much confusion. Furthermore, available studies evaluating the ability of HbA1c to detect IGT and diabetes in PCOS have found that the test has low sensitivity when compared with OGTT for the assessment of glucose tolerance[66,67]. Finally, the diagnostic accuracy of HbA1c in detecting T2D has recently been questioned, with some investigators arguing strongly in favor of OGTT for this procedure[77].

There is, moreover, much discordance among experts regarding the frequency of glycemic status assessment, ranging from yearly to on a five-year basis depending on the coexistence of additional factors. All the latter recommendations are illustrated in Table 2. However, from a pathophysiological point of view, PCOS women with IFG constitute a different subgroup from those with IGT. In fact, data derived from a healthy population have shown that isolated IFG is usually observed in subjects with predominantly hepatic IR and normal muscle insulin sensitivity, whereas individuals with isolated IGT have normal to slightly reduced hepatic insulin sensitivity and moderate to severe muscle IR[78]. Accordingly, those with IFG may represent the general population of women prone to T2D development, whereas subjects with IGT may compose that group of patients in whom dysglycemia occurs as a consequence of hyperandrogenemia. Today, in fact, copious documentation of the detrimental effects of androgens on muscle insulin sensitivity in lean women with PCOS is underway[1].

The conversion rate from normal glucose homeostasis to IGT or from IGT to T2D in PCOS has been estimated to range from 2.5 to 3.6% annually over a period of 3–8 years [79-81]. These conversion rates are lower than those observed in individuals with IGT in the general population, who seem to develop T2D at rates of approximately 7% annually[82]. This discrepancy may be related to the fact that the underlying mechanisms of T2D development in PCOS are different from those found in healthy individuals. In fact, in non-PCOS women, the degree of insulin sensitivity progressively deteriorates, thus leading almost inevitably to T2D. In contrast, in women with PCOS, a diversity of IR values has been observed over time, appearing to improve in lean subjects and worsen only when obesity is present[52,53]. Non-linear progression to T2D is therefore quite frequent in subjects suffering from PCOS, and

this strongly correlates with their degree of adiposity. This notion is further supported by Celik *et al*[83] who showed that obese women with PCOS had a 4-fold greater risk of converting from normal glucose tolerance to IGT. The latter finding was additionally corroborated by Rubin *et al*[4] who demonstrated that lean women with PCOS had an equal risk of progressing to T2D to that of controls.

RISK FACTORS FOR THE DEVELOPMENT OF T2D IN PCOS

Several parameters which could possibly mediate the risk of developing T2D in women with PCOS have recently been evaluated. With regard to PCOS phenotypes, the presence of the most severe form of PCOS, consisting of the conglomeration of chronic anovulation and elevated androgen concentrations (former NIH criteria), was found to be one of the strongest independent predictors of glucose concentrations after a 75-g OGTT in 254 women with PCOS following adjustment for several confounders, including age, waist-to-hip ratio (WHR), and BMI[61]. However, this finding has not since been replicated[13].

Based on the well-known developmental origins of health and disease, an inverse association between birth weight and T2D risk seems highly likely. Indeed, low birth weight has been associated with PCOS diagnosis later in life, with a birth weight < 2.5 kg conferring a 76% higher likelihood of developing PCOS[84]. In a similar manner, age of menarche has been related to dysglycemia in PCOS. Indeed, PCOS women with IGT were observed to have significantly earlier menarche age (11.9 ± 1.6 years) compared to obese women with PCOS and normal glucose tolerance (12.4 ± 1.7 years) in a cross-sectional study of 121 Italian PCOS women. However, the number of subjects with T2D was too small for any correlation to be established[3]. Of note, a single-center cohort study demonstrated that a higher number of births decreased the risk of T2D in women with PCOS, corroborating a potential prophylactic effect of parity in T2D[4]. In the same context, the impact of lactation on T2D development may be hypothesized. In fact, obesity in women with PCOS is a risk factor for impaired lactogenesis and increases the risk for reduced breastfeeding initiation and duration [85]. Furthermore, since lactation is crucial for women's post-gestational metabolic health[86], the presence of abnormal lactational function might enhance the risk for T2D in this population, even though substantial data to support this hypothesis are to date lacking. Finally, a positive link has been proposed between family history of T2D and T2D risk in PCOS; this hypothesized association is based on a defect in the first phase of insulin secretion in PCOS women with a first degree relative suffering from T2D, in contrast to BMI-matched PCOS patients without such a history[87].

Indisputably, the impact of environmental factors on T2D development is major. Endocrine disruptors constitute an emerging environmental threat, and a role for endocrine disrupting chemicals in exacerbating PCOS pathology has been proposed for over 20 years, with bisphenol A (BPA) being significantly associated with measures of IR and BMI in women with the syndrome[88]. Moreover, a positive feedback loop between BPA and hyperandrogenemia has been shown in PCOS and, therefore, BPA exposure has been particularly incriminated in PCOS pathophysiology[89]. Furthermore, the role of advanced glycation end-products (AGEs) in the pathophysiology of T2D development in PCOS is another issue that has gained ever more attention over time. AGEs are products of non-enzymatic glycation and oxidation (glycoxidation) of proteins and lipids in both hyperglycemic and euglycemic states. Thermally processed foods, mostly lipid- and protein-rich foods typical of Western diets, are responsible for the exceedingly high intake of exogenous AGEs, these remaining in the body and being incorporated covalently in different tissues. Dietary AGEs have been associated with subclinical inflammation and endothelial dysfunction both in patients with T2D and in unaffected individuals[90]. Of interest, concentrations of AGEs are elevated in women with PCOS compared to their healthy counterparts independently of the degree of obesity and IR[91]. They are positively associated with androgens and anti-Müllerian hormone levels and are localized in human polycystic ovaries. Based on all the above, a potential role of AGEs in the PCOS machinery has been suggested[90]. In women with PCOS, consumption of a diet high in AGEs was followed by a deterioration of IR and hyperandrogenemia, whereas elimination of AGEs was followed by a significant amelioration of these key parameters, even without a change in BMI[92]. On the other hand, the link of endocrine disruptors with human pathophysiology should be interpreted with caution, since their actions are exerted in an non-monotonic pattern[93].

Three of the most common addictions have been incriminated in PCOS pathophysiology and, possibly, in the development of T2D, namely, smoking, caffeine, and alcohol. Regarding tobacco use, a cross-sectional study of 309 women with PCOS identified a significant positive correlation between lipid concentrations and years of smoking, while IR was significantly higher in smokers with PCOS compared to non-smokers, despite the absence of a significant association between HOMA-IR and pack-years among the participants[94]. In addition, HOMA-IR and fasting insulin concentrations were higher in smokers with PCOS compared with their non-smoker counterparts in a German study of 346 women with PCOS, although the latter analysis was not adjusted for BMI or age[95]. Regarding caffeine, higher intake has been associated with worse reproductive outcomes in women with PCOS, but no study to date has found any association with glucose homeostasis[96]. Finally, a positive association between alcohol consumption and PCOS has been documented[97].

Another risk factor contributing to impaired glucose homeostasis may be vitamin D deficiency. Regarding PCOS, lower 25-hydroxy-vitamin D [25(OH)D] concentrations have been reported in PCOS patients compared to those in controls, with vitamin D deficiency [25OHD < 20 ng/mL] being associated with higher fasting glucose and insulin concentrations, as well as IR, assessed by OGTT[98]. A meta-analysis combining data from 11 placebo-controlled randomized trials evaluating the effects of vitamin D supplementation on glucose homeostasis in 601 patients with PCOS (89% of Asian descent) found that daily supplementation with small doses of vitamin D was able to significantly lower HOMA-IR index (daily supplementation effect -0.30; P = 0.0018, low-dose supplementation effect −0.31; P = 0.0016)[99]. However, both studies failed to report data regarding the relationship between vitamin D deficiency and T2D.

Taking a more holistic approach, other emerging factors in T2D development are sleep quality and mood disorders. It is well-known that women with PCOS, even after controlling for obesity, tend to have a higher prevalence of sleep disturbances, such as reduced sleep efficiency, amount of time spent in rapid eye movement (REM) sleep, as well as non-REM sleep, and difficulty in falling asleep and maintaining sleep[100]. Moreover, the prevalence of obstructive sleep apnea is higher in women with PCOS compared to non-PCOS [odds ratio (OR) 3.83; 95%CI: 1.43-10.24][101] and is associated with higher fasting insulin levels, HOMA-IR index, HbA1c, and glucose area under the curve[102]. On the other hand, the latter findings warrant caution, since a high likelihood for selection bias is considered plausible[101]. Depression and mood disorders have been associated with IR, obesity, and T2D in multiple studies[103]. In addition, depression and mood disorders have been commonly diagnosed in women with PCOS[104,105]. Despite the theoretical possibility of an association between these two conditions, no study has evaluated such an association to date.

In recent years, the role of the gut microbiome in metabolic abnormalities, including PCOS, has been explored[106]. It was thus inevitable that an effort would be made to improve some of the features of the syndrome by intervening in the microbial population with probiotics and synbiotics. The latter intervention was shown to confer beneficial effects on body weight, fasting plasma glucose and insulin, reproductive hormones, and hirsutism, while longer duration of treatment also seemed to be more efficacious[107].

A major factor that may eliminate or reduce T2D risk in PCOS is prescription of appropriate drugs. Oral contraceptives have been the mainstay of treatment for irregular menses, hirsutism, and acne in women with PCOS with exceptional success rates. Some studies have, however, suggested an increased risk for T2DM with this strategy[4], albeit a meta-analysis of published trials identified only a minor increase in fasting insulin concentrations[108]. By contrast, given the significance of IR in the pathophysiology of the syndrome, it comes as no surprise that metformin has been the most commonly used medication to prevent or treat the metabolic abnormalities in women with PCOS[109]. Yet, despite the high expectations, metformin combined with lifestyle changes appeared to produce only a small reduction in BMI (-0.73 kg/m^2). This was, namely, a decrease in subcutaneous adipose tissue volume and an improvement in menstrual cyclicity compared to lifestyle interventions alone, these as seen in a meta-analysis of published randomized controlled trials[109]. It is, however, of note that most studies were small in sample size and the risk of bias was deemed high by the authors. Patients with concurrent diabetes were excluded in most studies, not allowing for safe conclusions to be drawn in this regard. On the other hand, metformin significantly reduced the risk for gestational diabetes (pooled OR 0.20, 95%CI: 0.12-0.1, P < 0.001), early pregnancy loss (pooled OR 0.28, 95%CI: 0.10-0.75, P = 0.01), and preterm delivery (pooled OR 0.33, 95%CI: 0.18-0.60, P = 0.0003), with significant heterogeneity between studies[110]. These outcomes did not differ between patients treated prior to pregnancy and those treated throughout the duration of their

pregnancy[111].

In addition to metformin, PPAR-γ agonists, such as rosiglitazone (not used currently due to heart failure risk) and pioglitazone, which are potent insulin sensitizers, have been studied in women with PCOS, exhibiting significant improvements in fasting blood glucose and glucose tolerance[25]. In 2017, a meta-analysis of 11 randomized controlled trials comparing the effects of metformin and pioglitazone in 643 subjects with PCOS identified a number of promising effects on reproductive and esthetic outcomes and, as expected, metformin seemed to ameliorate BMI more effectively than pioglitazone[112]. Other T2D medications have been studied in women with PCOS recently, including the SGLT-2 inhibitor empagliflozin, which produced significantly more weight loss compared to metformin in a small study of non-diabetic women[113]. Exenatide[114,115] and liraglutide[116] appeared to improve several glucose homeostasis parameters in non-diabetic women with PCOS more efficaciously than metformin, while their addition to metformin seemed to provide incremental benefits in that regard[116,117]. Finally, orlistat has been studied extensively in obese women with PCOS and was found equally efficacious to metformin in producing weight loss and metabolic improvements[118].

Even though supplement use has increased greatly in the past few years, supplementation with minerals, trace elements, and other supplements is, in general, still controversial. In the case of chromium supplementation in PCOS, two meta-analyses of published trials found that BMI, fasting insulin, free testosterone[119] HOMA-IR, and HOMA-β[120] seemed to improve. In addition, supplementation with omega-3 fatty acids at doses of 900-4000 mg daily appeared to improve HOMA-IR index in a meta-analysis of nine small randomized controlled trials, but no data were available on risk for T2D[121]. Myoinositol, an amino acid with potentially beneficial effects in women with PCOS, has been studied with regard to glucose metabolism, and a small positive impact on fasting insulin and HOMA-IR was found in a 2017 meta-analysis of controlled trials, without any effect on glucose concentrations or BMI[122]. The factors described above and their relationship to development of dysglycemia in PCOS are illustrated in Figure 3.

T2D IN POSTMENOPAUSAL WOMEN WITH PCOS

Based on the aforementioned data, a diagnosis of PCOS during the reproductive age places a woman at increased risk for T2D in later, post-reproductive life[123]—a risk which is further augmented if the issue of dysregulated glucose metabolism during transition to menopause is taken into account. The risk is even greater if the woman enters menopause before the age of 45[124]. A recent meta-analysis of 23 cohort studies[47] assessed the long-term cardiovascular disease (CVD) risk in women with PCOS, including T2D risk. Women with a history of PCOS demonstrated a 3-fold increased risk of T2D (RR 3.00; 95%CI: 2.56-3.51) compared to non-PCOS women. However, the studies included were quite heterogeneous in terms of the participants' age. Very few of them included postmenopausal women, either as a single or a mixed population[8,125,126]. Another shortcoming was the heterogeneity in PCOS diagnosis among studies.

A recent prospective cohort study evaluated the risk for development of T2D in 27 PCOS women (defined by the NIH criteria) after 24 years of follow-up (mean age at baseline: 29.5 ± 5.3years; mean age at the end of follow-up: 52.4 ± 5.4 years) in comparison with age-matched non-PCOS controls (n = 94). The incidence of T2D in the former group was 19% compared to 1% in the latter ($P < 0.01$)[8]. Interestingly, the development of T2D was independent of lifestyle factors. Although all PCOS women with T2D were obese and had a higher BMI and WHR than non-PCOS individuals, the increases in BMI and WHR per year were comparable between groups during follow-up. However, an older prospective study including 35 postmenopausal women with PCOS (mean age 70.4 ± 5 years, range 61-79), diagnosed with the Rotterdam criteria, did not find any difference in T2D incidence compared to age-matched controls after 21 years of follow-up[125]. Moreover, a retrospective cohort study published in 2000, which included 319 PCOS women (mean age 56.7 years, range 38-98; 81% postmenopausal; PCOS diagnosis based on medical records) and 1060 age-matched controls, showed a 3-fold increased risk of T2D in the PCOS group (OR 2.8; 95%CI: 1.5-5.5) after a mean follow-up time of 31 years (range 15-47). However, this risk was not significant after adjustment for BMI (OR 2.2; 95%CI: 0.9-5.2)[126].

In general, whether the increased T2D risk exists in both obese and non-obese PCOS has not as yet been fully elucidated. A recent meta-analysis by Zhu *et al*[10] that

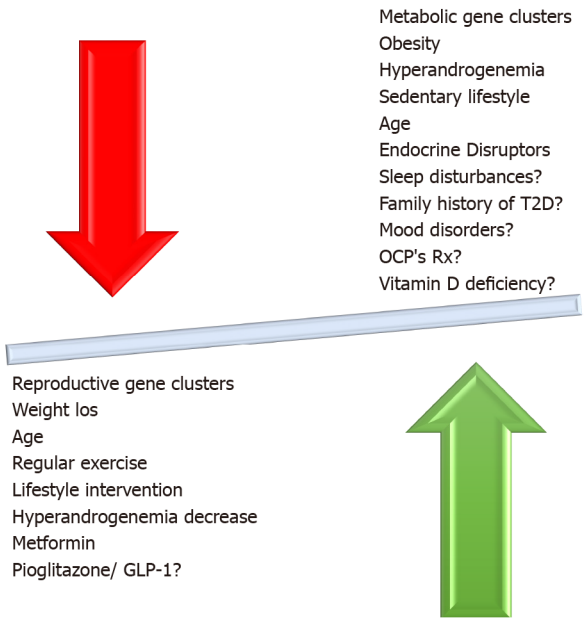

Figure 3 The interaction of positive (green arrow) and negative (red arrow) factors affecting dysglycemia in women with polycystic ovary syndrome. T2D: Type 2 diabetes mellitus; GLP-1: Glucagon-like peptide 1.

assessed the risk of T2D in non-obese PCOS compared to non-obese control women showed an increased risk in this PCOS subpopulation, although to a lesser extent compared to obese PCOS (five studies; OR 1.47; 95%CI: 1.11–1.93). The authors additionally reported an increased prevalence of IR, IGT, and atherogenic dyslipidemia in non-obese PCOS compared to non-obese non-PCOS women. It must be underlined that the PCOS populations in the included studies were all premenopausal [10]. Of note, a prospective cohort study of this meta-analysis assessed the incidence of T2D at the age of 46 years in a cohort of 279 women with both oligoamenorrhea and hirsutism at the age 31 years, who had been defined as "PCOS". This cohort was compared with 1577 women, without oligoamenorrhea and hirsutism, who served as controls. Women with PCOS and BMI > 25 kg/m^2 demonstrated a 2.5-fold increased risk of T2D compared to non-PCOS (OR 2.45; 95%CI: 1.28–4.67). It is notable that no such risk was identified in PCOS women of normal weight[127]. A very recent case-control study (1136 PCOS patients, aged 15 to 44 years, and 5675 controls) showed an increased risk of T2D in PCOS independently of BMI (adjusted OR in the entire cohort 2.36, 95%CI: 1.79–3.08; OR in non-obese PCOS 2.33, 95%CI: 1.71–3.18; OR in obese PCOS 2.85, 95%CI: 1.59–5.11)[128].

Aside from BMI, other factors also play an important role in the incidence of T2D in postmenopausal women with a history of PCOS. The increased ovarian androgen production and IGT observed in premenopausal women with PCOS cases seem to persist after menopause transition. On the other hand, IR and hyperinsulinemia may improve in women with PCOS during their post-reproductive years, although data are still inconsistent as to this hypothesis[129]. Furthermore, the severity of IR is also dependent on the PCOS phenotype, since hyperandrogenemia is related to a more severe metabolic dysfunction[65].

Therefore, although transition to menopause is associated with dysregulation of glucose metabolism[130], current evidence is at present too weak to support the existence of increased T2D risk in postmenopausal women with a history of PCOS compared to those without. There are currently too many confounding factors and variables, such as PCOS definition, sample size, and the precise effect of BMI and aging, to accurately determine the actual impact, if any, of a PCOS diagnosis on T2DM risk after transition to menopause.

MANAGEMENT OF T2DM RISK IN PCOS

Lifestyle intervention, including diet modification and regular exercise, still remain the mainstay of treatment in reducing T2D risk in women with PCOS, especially those who are obese or overweight. According to a recent meta-analysis of 19 randomized

controlled trials (RCTs), systematic dietary intervention is superior to advice, usual diets, or no treatment with regard to HOMA-IR [mean difference (MD) -0.78, 95%CI: -0.92 to -0.65], fasting plasma insulin (FPI) (MD -4.24 mIU/L, 95%CI: -5.37 to -3.10), FPG (MD -0.11 mmol/L, 95%CI: -0.17 to -0.04 mmol/L), as well as BMI (MD -1.01 kg/m^2, 95%CI: -1.38 to -0.64) and waist circumference (WC) (MD -3.25 cm, 95%CI: -5.29 to -1.22)[131]. Subgroup analysis showed that the Dietary Approaches to Stop Hypertension (DASH) diet is more effective in HOMA-IR and FPG reduction than a low-carbohydrate diet (LCD), but with comparable efficacy regarding FPI concentrations. With respect to BMI and body weight, a calorie-restricted diet is more beneficial than either DASH or LCD. All dietary patterns seem to have comparable efficacy regarding WC[131]. Moreover, data from RCTs in PCOS women have shown that LCD is quite effective in reducing BMI [standardized MD (SMD) -1.04, 95%CI: -1.38 to -0.70) and HOMA-IR (SMD -0.66, 95%CI: -1.01 to -0.30) compared to a regular diet according to a recent meta-analysis[132]. In addition, a low glycemic index diet could also be the first-line approach in PCOS patients, since it effectively reduces HOMA-IR (-0.78, 95%CI: -1.20 to -0.37), WC (-2.81 cm, 95%CI: -4.40 to -1.23), and total testosterone concentrations (-0.21 nmol/L, 95%CI: -0.32 to -0.09) compared to a high glycemic index diet[133]. Although the evidence in PCOS populations is limited, the Mediterranean diet (MedDiet) compared to the typical Western diet is also effective in reducing HOMA-IR (MD -0.42, 95%CI: −0.70 to −0.15), FPG (MD -2.98 mg/dL, 95%CI: -4.54 to -1.42), and FPI (-0.94, 95%CI: -1.72 to -0.16) compared to a usual diet. The MedDiet is also associated with a lower tendency to develop T2DM (RR 0.81; 95%CI: 0.61-1.02) and a reduction in CVD events related to metabolic syndrome[133].

The beneficial effects of structured exercise programs in metabolic syndrome, obesity, T2D, and CVD prevention and treatment are well-known[134]. Interventions consisting of lifestyle modifications in women with PCOS produce substantial improvements in glucose homeostasis and reproductive outcomes as well. These benefits are equally significant as those achieved by metformin[135]. A negative energy balance of approximately 30%, aiming to achieve an energy deficit of 500-750 kcal per day, is able to produce significant amounts of weight loss in women with PCOS. The addition of any amount of exercise, whether aerobic or anaerobic, confers additional beneficial effects on glucose homeostasis[25]. High-intensity interval training (achieving 90%-95% of the individual's maximum heart rate) three times a week for ≥ 10 wk is effective in reducing HOMA-IR (MD -0.57, 95%CI: -0.98 to -0.16) and BMI (MD -1.90, 95%CI: -3.37 to -0.42) in women with PCOS, according to a recent meta-analysis[136]. In cases with morbid obesity, such as those with BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with metabolic comorbidities, bariatric surgery should be considered[1]. Indeed, bariatric surgery can reduce the risk of T2D by 91% (RR 0.09, 95%CI: 0.03-0.32). The mean reduction in BMI is -14.51 kg/m^2 (95%CI: -17.88 to -11.14). It also ameliorates menstrual disturbances and hirsutism in PCOS patients [RR 0.23 (95%CI: 0.13-0.43) and 0.47 (95%CI: 0.28-0.79), respectively][137].

Metformin is the most commonly used insulin sensitizer in women with PCOS, especially in those who are obese or overweight. According to the aforementioned meta-analysis, diet is superior to metformin with regard to weight loss, but comparably efficacious in improving glucose homeostasis (HOMA-IR, FPG, and FPI) [131]. In a recent meta-analysis of 12 RCTs, metformin was superior to placebo in reducing BMI [weighted MD (WMD) -1.25, 95%CI: -1.60 to -0.91] and WC (WMD -1.41, 95%CI: -2.46 to -0.37). There were no differences between groups with regard to HOMA-IR, FPG, and FPI[138].

For women who are intolerant to metformin, thiazolidinediones (TZDs) constitute another class of insulin sensitizers that have been evaluated in women with PCOS. Rosiglitazone and pioglitazone, the two commonly used TZDs, are effective in improving IR and IGT, as well as mensural cyclicity, in PCOS patients. However, weight gain, increase in transaminase levels, and potential teratogenic effects limit their use in these patients[1]. Compared to metformin, pioglitazone is superior with respect to menstrual cycle improvement and ovulation but inferior regarding hirsutism score. Both agents are equally effective in reducing HOMA-IR, FPG, and FPI, as mentioned above[112].

Glucagon-like peptide 1 (GLP-1) receptor agonists (GLP-1-RAs) have also been tested in women with PCOS. A recent meta-analysis of eight RCTs (four with exenatide 10 μg twice a day; four with liraglutide 1.2 mg/d), compared their efficacy with that of metformin in women with PCOS. The study showed that GLP-1-RAs were more effective in improving HOMA-IR (SMD -0.40, 95%CI: -0.74 to -0.06) and reducing BMI (SMD -1.02, 95%CI: -1.85 to -0.19) and WC (SMD -0.45, 95%CI: -0.89 to -0.00). No difference between GLP-1-RAs and metformin in FPG and FPI was observed either between exenatide and liraglutide[139].

Finally, in cases of post-menopausal women younger than 60 years and/or within 10 years since their last menstrual period who present with severe vasomotor symptomatology, especially those with early menopause (< 45 years of age), menopausal hormone therapy (MHT) may be of benefit, since it reduces T2D by up to 30%[140]. MHT exerts a beneficial effect on glucose homeostasis in women both with and without T2D. In cases with T2D and low CVD risk, oral estrogens may be considered [141]. In obese women with T2D or with moderate CVD risk, transdermal 17β-estradiol is the preferred treatment, along with a progestogen with minimal effects on glucose metabolism, such as progesterone, dydrogesterone, or transdermal norethisterone. However, this favorable effect on glucose homeostasis is dissipated after MHT discontinuation. In any case, MHT is not recommended for the sole purpose of T2D prevention or treatment[140,141].

It is thus clear that weight loss, preferably with LCD and a low glycemic index diet low in AGES, combined with vigorous exercise should be the first-line lifestyle intervention in overweight or obese PCOS patients due to their well-documented beneficial effects on glucose metabolism, although longitudinal data on T2D risk are thus far lacking. The MedDiet may be even more beneficial than a low glycemic index diet in CVD risk reduction, although the current evidence in PCOS patients is weak. Metformin may also be considered in cases of impaired glucose metabolism and oligo/amenorrhea. The choice of either BS, pioglitazone, or GLP-1-RA should be individualized and benefits should be weighed against costs.

CONCLUSION

The association of PCOS with increased T2D risk is relatively robust and thus should not be neglected in any woman with the syndrome. Despite the current heterogeneity of the data, the ever-changing nature of this disorder, and the uncertainty regarding the exact mechanisms regulating progression of dysglycemia in PCOS, there are several general principles that the clinician should implement in everyday practice. First, diagnosis and follow-up of dysglycemia should preferably be based on OGTT and not on FPG or HbA1c values. Second, the non-linear development of T2D in PCOS in non-obese women highlights the importance of maintaining an optimal weight in all women suffering from the syndrome. Third, menopausal women with a history of PCOS should be regularly evaluated since they may be at higher T2D risk, especially if they are obese. Fourth, a well-balanced diet coupled with regular exercise constitutes the most appropriate approach in every patient with PCOS. Metformin administration might ameliorate the biochemical and hormonal profile in PCOS and may be considered in patients in whom prior measures have failed to improve metabolic and ovulatory dysfunction. The use of pioglitazone, GLP-1-Ras, and/or MHT may be of value in selected cases.

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 January 15; 13(1): 27-36

DOI: 10.4239/wjd.v13.i1.27

ISSN 1948-9358 (online)

MINIREVIEWS

Management of diabetic foot ulcers and the challenging points: An endocrine view

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Author contributions: Doğruel H, Aydemir M, and Balci MK conceived and designed the study; Doğruel H and Aydemir M searched the literature; Doğruel H drafted the article; Aydemir M and Balci MK made critical revisions; all authors gave final approval for the article.

Conflict-of-interest statement: There is no conflict of interest.

Country/Territory of origin: Turkey

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Diabetic foot ulcers (DFU) are one of the most challenging complications of diabetes. Up to one-third of patients with diabetes mellitus (DM) may suffer from DFUs during their life. DFU is one of the leading causes of morbidity in patients with DM. The treatment period is challenging, and the recurrence rate of DFUs is high. Hence, establishing prevention strategies is the most important point to be emphasized. A multidisciplinary approach is necessary in the prevention and treatment of DFUs. Patients at risk should be identified, and prevention measures should be taken based on the risk category. Once a DFU is formed, the appropriate classification and evidence-based treatment interventions should be executed. Glycemic control, diagnosis and treatment of vascular disease, local wound care, diagnosis, and treatment of infection should be addressed along with the proper evaluation and management of general health status.

Key Words: Diabetic foot; Diabetic foot ulcer; Amputation; Diabetic foot infection

Core Tip: Diabetes mellitus is a chronic disorder with dramatic complications. Nearly one-third of patients with diabetes may suffer from foot ulcers during their life. A potentially preventable event usually has dramatic results. The prevention and management of diabetic foot ulcers (DFUs) necessitate a multidisciplinary approach. The most important approach is the prevention of the formation of DFU. Prevention measures should be implemented in a timely manner, and adequate treatment interventions should be executed immediately once it is formed.

Received: May 31, 2021
Peer-review started: May 31, 2021
First decision: June 23, 2021
Revised: July 18, 2021
Accepted: December 11, 2021
Article in press: December 11, 2021
Published online: January 15, 2022

P-Reviewer: Wu QN
S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

Citation: Doğruel H, Aydemir M, Balci MK. Management of diabetic foot ulcers and the challenging points: An endocrine view. *World J Diabetes* 2022; 13(1): 27-36
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/27.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.27

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a global health problem in the last decades[1]. DM has several complications that affect not only life expectancy but also the quality of life[2,3]. Diabetic foot ulcers (DFU) are one of the most challenging complications of DM. Up to one-third of diabetic patients may suffer from DFUs during their life[4,5]. The global prevalence of DFUs is reported at 6.3%, with DFUs being more common in men than women and in type 2 DM than type 1 DM[6]. The recurrence rate of DFUs is also high. The value reaches 40% within 1 year and 65% within 3 years[4]. Hence, studies should focus on establishing prevention strategies against DFU[4,5].

PATHOPHYSIOLOGY AND PREDISPOSING FACTORS

Peripheral artery disease (PAD) and diabetic neuropathy (DNP) are well-known chronic complications of diabetes[7]. Along with immune dysfunction, PAD and DNP are the main pathophysiological factors that predispose to DFUs[8]. DFUs are associated with DM duration, the presence of DNP, and PAD[9]. DNP is present in 80% of patients with DFUs, and it facilitates ulcer formation by causing decreased pain and pressure sensation. DNP also promotes the formation of anatomic deformities, such as prominent plantar metatarsal heads, hammertoes, Charcot foot, *etc.*[4,10]. Patients with diabetes should be assessed for DNP periodically after the diagnosis of type 2 DM and after the fifth year of type 1 DM. Pain, burning, and numbness should be questioned. Small fibers (by pinprick test and temperature sensation), large fibers (by vibration perception and 10 g monofilament test), and protective sensation (by 10 g monofilament test) should be tested. The tests predict the risk of complications besides screening the dysfunction[7,11,12].

Nearly half of the patients with DFUs have PAD, which is significantly associated with the increased risk of adverse limb events[13]. Vascular symptoms, including reduction in effort capacity, leg fatigue, and claudication, should be assessed. All peripheral pulses should be palpated together with an assessment of extremity appearance and warmth to evaluate perfusion[8,13]. Patients should also undergo the ankle-brachial index (ABI) testing as a part of the examination. The normal value of ABI is between 0.9 and 1.3, which is higher than 1.0[13,14]. A high ABI may be measured falsely in the presence of vascular calcifications[13]. Toe-brachial index (TBI) measurement is also recommended, especially in combination with ABI and arterial Doppler study. The diagnosis of PAD is unlikely in the presence of triphasic Doppler waveforms when the TBI is ≥ 0.75, and the ABI is between 0.9-1.3[13]. In addition, disrupted blood flow may be present at the microvascular level despite the intact or well-treated macrovascular component[15]. Dysfunctional signs of blood flow at the microvascular level can be detected by laser Doppler flowmetry[16]. Furthermore, DM causes immunological dysfunctions at the cellular level, leading to poor healing response and susceptibility to infections[8,17].

CLINICAL SIGNIFICANCE

DFUs are a serious healthcare problem globally. A potentially preventable event, such as a minor trauma, usually has dramatic results. DM remains the primary cause of nontraumatic lower-limb loss worldwide[18-20]. DFUs pose a serious financial burden worldwide, and nearly one-third of expenses for DM is estimated to be for DFUs[21-23]. The presence of DFU is associated with the increased risk of mortality in DM, and this association is stronger than the presence of any macrovascular disease alone[3,24]. The five-year survival rate in patients presenting with DFUs is poorer than that associated with the most common cancers[21]. Therefore, the best approach in the management of DFUs is the implementation of preventive measures based on the risk

class[7,10,25].

IDENTIFICATION AND FOLLOW-UP OF PATIENTS AT RISK

DNP, PAD, foot deformity, and medical history of DFU are the most important risk factors for new DFU formation. These factors are the shadows of the coming event, which is DFU if the preventive measures are not applied in time[4,10,26]. Poor glycemic control, chronic kidney disease (especially dialysis), and smoking are also among the risk factors[7,8]. Diabetic patients should be categorized based on the risk of developing DFU. Thus, the risk factors for DFUs must be screened at least annually [7,12]. The risk classification system developed by the International Working Group on the Diabetic Foot (IWGDF) is useful in daily clinical practice (Table 1)[13].

A diabetic patient with very low risk (IWGDF group 0) must be examined annually for DNP and PAD. The patients who have a higher risk (IWGDF group 1-3) should be examined more frequently (Table 1), and preventive measures should be executed (Table 2)[7,13]. Patients who have moderate-to-high risk should wear therapeutic shoes to reduce plantar pressure and the risk of ulceration. Pre-ulcerative lesions, abundant callus, stinging toenails, and fungal infections (tinea pedis, onychomycosis, *etc*.) should be treated properly. Surgical interventions should be performed to fix deformities, if necessary[7,10,13]. The patient's feet with DNP should be inspected every visit, and the patients at risk should be encouraged and educated about self-care and preventive measures[7].

CURRENT EVIDENCE FOR PREVENTION

Several randomized clinical trials (RCT) evaluated the primary prevention strategies of DFUs, but none of them were high-quality research[27]. Conducting RCT to determine the primary prevention strategies and evaluate their efficacy is a considerable challenge, given that numerous patients and a long follow-up period will be required [21]. On the other hand, conducting RCT on the prevention of ulcer recurrence is technically easier because the recurrence rate is high[4,21]. Suitable therapeutic footwear with appropriate pressure distribution prevents recurrence or worsening of plantar foot ulcers, with high-quality evidence[7].

DNP and PAD are the major predisposing factors of DFU development[28,29]. Thus, the neurosensory and vascular systems of the extremities must be protected to prevent or delay the development of DFUs. The onset and progression of diabetic microvascular complications (retinopathy, nephropathy, and neuropathy) can be delayed by intensive glycemic control. This finding has been shown in type 1 DM, but the current evidence in type 2 DM is weak[30-32]. However, no specific therapeutic agent or approach other than glycemic control can modify the progression of microvascular complications[7,10].

PAD is one of the macrovascular complications of diabetes. The benefit of intensive glycemic control on macrovascular complications in diabetics has not been shown in RCTs, but several epidemiological analyses reported a correlation between an increased rate of cardiovascular disease (CVD) and chronic hyperglycemia[33-35]. The benefit of intensive therapy could not be shown in three large RCTs comparing intensive and conventional therapies in terms of cardiovascular benefits in patients with longstanding DM[36,37]. Unlike these studies, in a research investigating the effect of glycemic control on complications in newly diagnosed DM, the benefit of intensive glycemic control on CVD was shown after a 10-year follow-up on the post-interventional period[38]. The management of other CVD risk factors is particularly important in the prevention or delay of PAD and other macrovascular complications in patients with DM. Smoking cessation, effective treatment of hyperlipidemia and hypertension, weight loss, appropriate nutrition, and exercise habits are important points that should be emphasized in every patient with DM. Exercise should be considered with caution if the patient is in the risk group for DFU. Patients in the low- or moderate-risk group should be advised exercises that increase the motion of foot and ankle, relieve pressure, and decrease neuropathic symptoms. Patients in the risk group should avoid long walks, exercises that increase the pressure on the soles of feet, activities with a risk of trauma, and wearing inappropriate shoes[13].

Table 1 International Working Group on the Diabetic Foot risk classification system

Group	Definition	Ulcer risk	Screening
0	No LOPS and no PAD	Very low	Once a year
1	LOPS or PAD	Low	Once every 6-12 mo
2	LOPS + PAD or LOPS + FD or PAD + FD	Moderate	Once every 3-6 mo
3	LOPS or PAD with one or more of the following: (1) History of a foot ulcer; (2) A lower extremity amputation (major or minor); and (3) ESRD	High	Once every 1-3 mo

LOPS: Loss of protective sensation; PAD: Peripheral artery disease; FD: Foot deformity; ESRD: End-stage renal disease.

Table 2 Preventive measures for diabetic foot ulcers

	Preventive measures
1	Avoid smoking
2	Avoid walking barefoot/in socks without shoes/in thin-soled slippers
3	Avoid hot ground and hot sand
4	Inspect both feet and inside the shoes daily
5	Wash the feet daily (carefully dry especially between the toes)
6	Test water temperature before bath
7	Lubricate dry skin and avoid chemicals
8	Cut the toenails straight
9	Do not remove callus
10	Wear snug shoes (customize if feet have deformity)
11	Change the socks daily

CURRENT TECHNOLOGICAL OPPORTUNITIES FOR MONITORING

The recurrence rate of DFUs is also extremely high in patients who are under follow-up in specialized centers. Thus, systems that facilitate recognition of the early signs of DFU formation must be developed. Patients can refer to health care providers early, and preventive and/or therapeutic appropriate strategies can be executed on time[42]. Risky conditions for DFU formation, such as early signs of inflammation and pressure-induced plantar tissue stress by current technological opportunities, can be screened and followed-up[29]. The available technological devices had been invented for this purpose; these devices include instruments for daily monitoring plantar temperature, socks that enable temperature monitoring continuously, socks that monitor plantar pressure, smart insoles to screen sustained plantar pressure, alarm systems that warn patients to wear offloading devices, activity monitoring devices, *etc*.[29].

POINTS TO BE CONSIDERED IN DFU MANAGEMENT

DFU is the major cause of nontraumatic lower extremity amputations (LEA), worldwide[20,39]. Once DFU occurs, the management strategies should be implemented without delay. Numerous studies emphasized the importance of a multidisciplinary team approach in the management of these patients[20,40,41]. The multidisciplinary team should focus on four major points; glycemic control, diagnosis and treatment of vascular disease, evaluation and local management of wound, diagnosis, and treatment of infection[41].

Glycemic control

The importance and role of adequate glycemic control for delaying or preventing chronic complications of DM are discussed above. Although RCTs have shown an association between intensive glycemic control before DFU formation and the low risk of LEAs, to our knowledge, the role of glycemia in the management of active DFU has never been studied in RCTs[42,43]. Considering the known negative effect of hyperglycemia on wound healing and immune defense, hyperglycemia may be associated with negative consequences in patients with DFUs[8,17,44]. Several meta-analyses of observational studies addressed this point[43,45,46]. Margolis *et al*[46] published a meta-analysis of five observational studies including DFUs. Glycemic control was not associated with wound healing according to this study. The other two meta-analyses reported that the high fasting plasma glucose and Hba1c levels were associated with a high rate of amputations[43,45].

In addition to the effect of hyperglycemia on the wound healing process, hyperglycemia causes impaired immune functions and decreased response to infections[20,47]. American Diabetes Association (ADA) and American Association of Clinical Endocrinologists (AACE) recommend targeting glucose levels between 140-180 mg/dL without causing hypoglycemia in the majority of inpatients[48]. These levels should be aimed at patients with DFUs treated in inpatient setting.

An intercurrent illness (trauma, infection, surgery, *etc.*) causes impaired glycemic control in diabetics and necessitates adjustment of the therapy. Here, DM patients are predisposed to severe hyperglycemia, diabetic ketoacidosis, and nonketotic hyperosmolar state. Patients treated with noninsulin antidiabetics require insulin. ADA and AACE recommend insulin regimens for critically ill and noncritically ill hospitalized patients[48].

Several oral antidiabetics have other properties besides the glucose-lowering effect. For instance, canagliflozin, a sodium-glucose cotransporter-2 (SGLT-2) inhibitor, is associated with an approximately two-fold increased risk of LEA (primarily at the level of toe or metatarsal) in patients with type-2 DM and established CVD (or at risk for CVD) *vs* placebo[49]. On the other hand, in RCTs of empagliflozin and dapagliflozin, the risk of amputation was similar between the treatment and placebo arms [50]. An increased risk of LEAs was reported with canagliflozin, empagliflozin, and dapagliflozin (for toe amputations) in a pharmacovigilance study. This study relied on several LEA cases[51]. Recent meta-analyses found no associations between SGLT-2 inhibitors and increased LEA risk; however, Chang *et al*[53] compared the use of SGLT-2 inhibitors with other oral antidiabetics and reported that SGLT-2 inhibitors may contribute to the increased risk of LEA[49,52]. A study examining systematic reviews, which evaluated the adverse effects of SGLT-2 inhibitors, summarized the scarcity of high-quality systematic reviews on this topic[54]. To our opinion, SGLT-2 inhibitors may increase the risk of LEA in patients with DFU as a group effect. Conflicting data are available regarding this traffic; thus, exercising cautiousness is reasonable.

Vascular disease

The prevalence of PAD among DFU patients reaches 50%. The presence of PAD is significantly related to adverse limb events. All patients with DFU should be examined clinically for PAD. Doppler sonographic study should be performed with a combination ABI and/or TBI test. No single modality has been defined as optimal. Vascular imaging (and revascularization if PAD is present) should always be considered when the ulcer remains unhealed in 4-6 wk despite the appropriate treatment and normal condition (ABI and TBI)[7,13]. The threshold for performing vascular studies should be very low in DFU patients, especially for those who are unresponsive to treatment[55]. Based on the vascular structure and clinical conditions, surgical bypass or endovascular treatment can be applied as a revascularization therapy[15,55].

Local wound management

The first step in the treatment of DFUs is to classify the wound and assess the patient's medical condition. The depth and width of the ulcer, the presence of ischemia, and infection should be evaluated. Classification systems have been developed for DFUs (Table 3). Wound classification helps in the prediction of prognosis, along with determining the type and intensity of treatment[20,56,57]. All infected and nonvitalized tissues should be removed by surgical debridement, and the abscess should be drained, if present[58]. Other debridement methods, such as mechanical, enzymatic, and biological debridement, are available other than surgical procedures[20]. Surgical

Table 3 Classification systems of diabetic foot ulcers

Classifications system	The evaluated parameters
University of Texas System	Depth, infection, ischemia
Wagner	Depth, necrosis
PEDIS	Perfusion, extent, depth, infection, sensation
SINBAD	Site, ischemia, neuropathy bacterial infection, area, depth
Threatened limb classification: WIfI	Wound characteristics, ischemia, foot infection
IWGDF/IDSA system	Clinical manifestations, the severity of infection, PEDIS grade

IWGDF: International Working Group on the Diabetic Foot; IDSA: Infectious Disease Society of America.

debridement is the most effective and preferred method[20,58].

Post-debridement wound care is vital. Further tissue injury should be avoided. Proper wound coverage and dressing are necessary. Negative pressure therapy can be used if the wound is clean. Wound characteristics are determinative of the dressing procedure. Pressure reduction is another important point for wound healing. Several available methods of mechanical offloading (cast walkers, wedge shoes, bed rest, *etc.*) are also applied. Surgical pressure reduction may be needed occasionally[20,56,57].

Management of infection

DFUs are predisposed to infection. The exact diagnosis of infection should be performed correctly the first time to manage the infection in DFUs. The classical manifestations of inflammation (warmth, erythema, tenderness, and swelling), extent of infection, involvement of deep tissues and/or bones, and presence of an abscess and/or fistula tract should be evaluated. The clinician should be acquainted with the clinical findings of necrotizing infections. Systemic manifestations of infection (including findings of systemic inflammatory response syndrome and sepsis) and hemodynamic status should also be assessed carefully along with the wound characteristics[58,59]. The presence of severe infection, extensive gangrene, necrotizing infection, deep abscess, compartment syndrome, and/or limb-threatening ischemia needs immediate consultation with a surgeon[59].

Most diabetic foot infections are polymicrobial. A wound specimen must be obtained for culture if no clinical sign of infection is observed[56]. However, the specimens for culture should always be collected in the presence of infection (especially in moderate-to-severe infection) before antibiotic administration[56,59]. Specimens for culture can be collected by aspiration of the abscess, curettage from the ulcer (after debridement), or biopsy during the surgical procedure (from deep tissue or bone) but not by superficial swab[58,59].

Empiric antimicrobial therapy should be considered in the presence of infection, and the selection of antibiotic should be based on clinical findings and the severity of infection[56,58,59]. An antibiotic regimen that covers gram-positive organisms only is preferable in antibiotic-naive patients with mild infections. In the case of antibiotic treatment in the last several weeks of, severely ischemic limb, or moderate-to-severe infections, the coverage of antibiotic therapy should include commonly isolated gram-negative organisms and anaerobes (in certain conditions) besides gram-positive organisms[59]. The clinical course and culture results should drive antibiotic therapy during follow-up[56,59].

POTENTIAL ADJUNCTIVE THERAPIES

In addition to all these interventions, several adjunctive therapies may help the healing of DFUs [negative pressure wound therapy (vacuum-assisted closure), skin grafts and substitutes, hyperbaric oxygen therapy, shock wave therapy, growth factors, autologous combined leucocyte, platelet, fibrin, and placental derived products][56,60]. No high-quality evidence supports the recommendation of these interventions without concern, and none of these treatments is an alternative to the best standard therapy[60].

CONCLUSION

A DFU is a challenging complication of diabetes that has become a global health problem. The treatment process is troublesome for the patient and healthcare team, and the treatment results are often unsatisfactory, especially in advanced cases. Moreover, the recurrence rate is high despite the healing of ulcer. DFUs are one of the leading causes of morbidity in diabetic patients.

DFUs are potentially preventable. Hence, strict implementation of primary and secondary prevention strategies should be implemented. However, the scarcity of high-quality evidence especially in establishing preventive measures for primary prevention is a challenge.

The multidisciplinary team approach is the cornerstone of the management of DFU. All the team members should be experienced in their field. The evidence-based standard follow-up and treatment algorithms should be applied without delay once an ulcer develops.

Geographic heterogeneity in terms of access to adequate healthcare equipment and experienced healthcare team, poor adherence of the patients, late reference to health care providers, difficulties in achieving adequate perfusion of ulcer, the presence of DNP, the impossibility of restoring sensation, and high recurrence rates are the featured challenging points in the management of DFU.

ACKNOWLEDGEMENTS

Preparation for publication of this article is supported by the Society of Endocrinology and Metabolism of Turkey.

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World Journal of Diabetes

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World J Diabetes 2022 January 15; 13(1): 37-53

DOI: 10.4239/wjd.v13.i1.37

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

High doses of catecholamines activate glucose transport in human adipocytes independently from adrenoceptor stimulation or vanadium addition

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Author contributions: Carpéné C designed the studies, performed the cell experiments, reviewed the literature and wrote and revised the manuscript; Grolleau JL obtained human biological resource; Boulet N isolated the cells for *in vitro* studies; Morin N was involved in data mining, contributed to the literature review and revised the manuscript.

Institutional review board statement: The study was approved by the I2MC Institutional Review Board: Institut des maladies métaboliques et cadiovasculaires.

Institutional animal care and use committee statement: Mice were housed and manipulated according to the INSERM guidelines and European Directive 2010/63/UE by competent and expert technicians or researchers in animal care facilities with agreement number A 31 555 04 and C 31 555 07. The experimental

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Abstract

BACKGROUND

When combined with vanadium salts, catecholamines strongly activate glucose uptake in rat and mouse adipocytes.

AIM

To test whether catecholamines activate glucose transport in human adipocytes.

METHODS

The uptake of 2-deoxyglucose (2-DG) was measured in adipocytes isolated from pieces of abdominal subcutaneous tissue removed from women undergoing reconstructive surgery. Pharmacological approaches with amine oxidase inhibitors, adrenoreceptor agonists and antioxidants were performed to unravel the mechanisms of action of noradrenaline or adrenaline (also named epinephrine).

RESULTS

In human adipocytes, 45-min incubation with 100 μmol/L adrenaline or noradrenaline activated 2-DG uptake up to more than one-third of the maximal response to insulin. This stimulation was not reproduced with millimolar doses of dopamine or serotonin and was not enhanced by addition of vanadate to the

protocol was approved by the local ethical committee CEEA nb122.

Conflict-of-interest statement: The authors declare no competing financial interests.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guideline.

Country/Territory of origin: France

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 18, 2021
Peer-review started: February 18, 2021
First decision: April 20, 2021
Revised: April 26, 2021
Accepted: December 28, 2021
Article in press: December 28, 2021
Published online: January 15, 2022

incubation medium. Among various natural amines and adrenergic agonists tested, no other molecule was more efficient than adrenaline and noradrenaline in stimulating 2-DG uptake. The effect of the catecholamines was not impaired by pargyline and semicarbazide, contrarily to that of benzylamine or methylamine, which are recognized substrates of semicarbazide-sensitive amine oxidase. Hydrogen peroxide at 1 mmol/L activated hexose uptake but not pyrocatechol or benzoquinone, and only the former was potentiated by vanadate. Catalase and the phosphoinositide 3-kinase inhibitor wortmannin inhibited adrenaline-induced activation of 2-DG uptake.

CONCLUSION
High doses of catecholamines exert insulin-like actions on glucose transport in human adipocytes. At submillimolar doses, vanadium did not enhance this catecholamine activation of glucose transport. Consequently, this dismantles our previous suggestion to combine the metal ion with catecholamines to improve the benefit/risk ratio of vanadium-based antidiabetic approaches.

Key Words: Human adipocytes; Amine oxidases; Insulin; Diabetes; Vanadium; Obesity

Core Tip: Our recent results indicated that the combination of catecholamines plus vanadium strongly stimulates glucose transport in rat adipocytes. We therefore proposed that catecholamine/vanadate salts could lead to the development of novel derivatives exhibiting potent insulin-like properties. Here, we found that adrenaline and noradrenaline stimulated glucose transport in human adipocytes but in a manner that was not dependent on and not enhanced by the presence of vanadate. Consequently, our previously proposed usefulness of the synergism of catecholamines/vanadium does not work in human fat cells. This might hamper the improvement of vanadium-based antidiabetic approaches, limited so far by toxicological issues.

Citation: Carpéné C, Boulet N, Grolleau JL, Morin N. High doses of catecholamines activate glucose transport in human adipocytes independently from adrenoceptor stimulation or vanadium addition. *World J Diabetes* 2022; 13(1): 37-53
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/37.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.37

INTRODUCTION

In a recent report, we demonstrated that catecholamines such as noradrenaline and adrenaline (also named norepinephrine and epinephrine) are capable of activating glucose transport in rodent adipocytes, essentially in the presence of vanadium[1]. These observations, providing the basis for novel research of vanadium/amine complexes exhibiting antidiabetic properties of the metal ions with less toxicological issues, needed further verification. Particularly, the demonstration of relevance to humans was lacking for this alleged insulin-like effect of high doses of catecholamines.

To extrapolate to humans our recent description of a stimulatory effect of catecholamines plus vanadium on glucose transport in rodent fat cells[1], we have reproduced our previous explorations in human adipocytes. Although cultured preadipocytes undergoing *in vitro* adipogenesis and immortalized cell lines have been successfully used to document the complex influence of pro- and antioxidants on insulin sensitivity[2,3], we have chosen to explore the effects of catecholamines in human mature adipocytes.

Since we have performed our previous observations on rodent white fat cells[1] and since white adipocytes store energy in adipose depots, it was of utmost importance to verify whether our findings are relevant for human mature adipocytes. Indeed, white adipocytes are not found in human adipose tissue because it is yellow mature fat cells that are present in fat depots, regardless of their anatomical location. The yellow coloration found in humans is attributed to the storage of natural lipophilic pigments

P-Reviewer: Ozkok E, Wu QN
S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

such as carotenoids, which are slowly metabolized. The white fat cells are somewhat specific of rodents, and this mere difference in the color is not the sole difference between fat cells from animal models and humans[4]. The sensitivity to vanadium regarding glucose accumulation in adipocytes is also different between rodents and humans, as recently reviewed[5]. Thus, an interspecies extrapolation step was mandatory prior to further development of our proposed combination of catecholamines plus vanadium for potential blood glucose-lowering approaches[1]. In the case of vanadium, its insulin-like properties originally described several decades ago[6,7] still requires the setting of novel administration forms to increase the benefit/risk ratio in diabetic patients[8,9].

The advantage of white adipocytes freshly isolated from young laboratory rodents previously used for the demonstration of the potential insulin mimicry of amines plus vanadium[1,10] is that such fat cells are highly sensitive to insulin stimulation of glucose metabolism and to the catecholamine stimulation of lipolysis. Interspecies comparative functional explorations of triacylglycerol synthesis (lipogenesis), triacylglycerol breakdown (lipolysis) and metabolite or adipokine release have shown multiple differences between rodent and human adipocytes, with human adipocytes being less metabolically active[11]. For example, the adrenergic stimulation of lipolysis is predominantly mediated by β_3-adrenergic receptor (β_3-AR) activation in rat and mouse, while it depends only on β_1-AR and β_2-AR activation in human adipocytes[4,12]. Similarly, the activation of α_2-ARs in human fat cells results in an antilipolytic response, which hardly occurs in rodent adipocytes since their equipment in α_2-ARs is rather limited[4]. Finally, the atrial natriuretic peptides are more lipolytic in human adipocytes than in the rodent ones[13,14].

All these considerations prompted us to test whether adrenaline and noradrenaline were activating glucose uptake in human adipocytes freshly isolated from pieces of abdominal subcutaneous adipose tissue removed during reconstructive surgery interventions in premenopausal women, as in[15,16]. When deciphering the effects of catecholamines in rat and mouse adipocytes, it has been evidenced that β-AR activation is not involved since catecholamines were able to activate glucose transport in fat cells from "beta-less" mice with triple knock-out of the subtypes of β-ARs[1]. In view of the above mentioned interspecies differences regarding adrenoreceptor equipment, it was necessary to perform such verification in human fat cells, and this implied the use of specific adrenergic agonists as reported in the following results.

Moreover, among the amines already reported to activate hexose uptake in human fat cells in the absence of insulin, it is worth mentioning benzylamine[17] and methylamine[15]. They are substrates of a copper-containing amine oxidase, the *AOC3* gene of which is highly expressed in human fat cells: the semicarbazide-sensitive amine oxidase (SSAO)[18], also known as primary amine oxidase[19], or vascular adhesion protein-1[20]. Benzylamine and methylamine have been included alongside insulin as positive controls of the hexose uptake activation in human adipocytes. As SSAO is not the sole amine oxidase present in adipocytes[21] [it coexists with monoamine oxidase (MAO-A, and to a lesser extend MAO-B)], their respective historical inhibitors semicarbazide and pargyline were also used. Also added in our control conditions was hydrogen peroxide, one of the reactive oxygen species (ROS) known to stimulate glucose uptake in fat cells[22] and is one of the historical insulin-mimetic compounds that act independently of insulin, while an excess of ROS hampers insulin action[3,23,24].

The following results, which can be considered as preclinical, will document that adrenaline and adrenaline stimulate hexose transport in human fat cells but in a manner that is not potentiated by sodium orthovanadate, not mimicked by β-AR or α_2-AR agonists and not hampered by SSAO and MAO inhibitors.

MATERIALS AND METHODS

Chemicals

(+/-)-Adrenaline (equivalent to epinephrine), (-)-noradrenaline (equivalent to norepinephrine), (-)-isoprenaline (equivalent to isoproterenol), dopamine, tyramine, benzylamine, sodium orthovanadate, collagenase A, human and bovine insulin and most of the other reagents were from Sigma-Aldrich-Merck (Saint Quentin Fallavier, France). [^{3}H]-2-deoxyglucose (2-DG) was from Perkin Elmer (Boston, MA, United States). The adrenergic agonists CL 316243 and BRL 37344 were given by Dr. Lafontan M. (Toulouse, France), while UK 14304 and RX 821002 were a gift from the late Dr. Paris H. (Toulouse, France).

Patients and adipose tissue surgery

Samples of abdominal subcutaneous adipose tissue were obtained with informed consent from a total of 34 women undergoing reconstructive surgery at the Department of Plastic Surgery (Rangueil Hospital, Toulouse, France). Mean age was 37 years (range: 18-59), and mean body mass index was 25.04 ± 0.65 kg/m^2 (range: 21-41). Adipose tissue samples were transferred to the laboratory in less than an hour after surgery and cut into small pieces then digested at 37 °C by collagenase under agitation. Preparations of buoyant adipocytes were obtained by filtration of the digested pieces through nylon stockings and two washes with Krebs–Ringer buffered at pH 7.5 with 15 mmol/L sodium bicarbonate, 10 mmol/L HEPES, supplemented with 3.5% of bovine serum albumin, as in[15]. No freezing/thawing sequences were inserted in the protocol for obtaining functional adipocytes, and 2-DG uptake assays were completed within 5 h after each surgical intervention. The study was in compliance with the INSERM guidelines and approved by the local ethics committee "Comité de Protection des Personnes Sud Ouest & Outre-Mer II" under the number DC-2014-2039.

Rodent adipocyte preparations

Male Wistar rats from Charles River (L'Arbresle, France) and mice of both genders from a mixed genetic background (129 Sv/ev, 129 Sv/J, FVB/N, C57BL/6J, and DBA/2) were housed in separate rooms at constant temperature (20-22 °C) and with a 12-h light-dark cycle. All the rodents had free access to food and water and were treated in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments)[25]. Only the mice that were considered as wildtype by Southern blot genotyping as described elsewhere[26] and were similar to those used as control for "β-less" mice in our previous study of catecholamine influence on adipocyte glucose transport[1] were euthanized after overnight fasting when 2- to 3-mo-old. Adipocyte preparations were obtained by collagenase digestion of perigonadic, retroperitoneal, perirenal and inguinal fat pads as previously described[1]. As for pieces of human adipose tissue, rodent fat pads were minced with scissors in Krebs-Ringer salt solution buffered at pH 7.5 and containing 3.5% fat-depleted bovine serum albumin.

Glucose transport assays

The only source of glucose for the cell preparations during glucose uptake assays was the non-metabolizable analogue [^{3}H]-2-DG, added at a final concentration of 0.1 mmol/L to fat cell suspensions as described previously[1]. Since the radioactive tracer (approximately 1300000 dpm/vial) was added for 10 min in the presence of fat cells after a 45 min preincubation period with the tested or reference agents, 2 mmol/L pyruvate was also present in the medium throughout the experiments for energy supply, as previously detailed[18]. Human or rodent fat cells were preincubated in 400 µL medium, then [^{3}H]-2-DG was added as 100 µL portions, and hexose uptake assays were stopped 10 min later with 100 µL of 100 µmol/L cytochalasin B. Then, 200 µL of cell suspension were immediately transferred to plastic centrifugation microtubes prefilled with dinonyl-phthalate (density 0.98 g/mL) before a 40 s spin to separate the buoying adipocytes from the medium as described previously[1,18]. The upper part of the tubes, containing radiolabeled hexose internalized in intact fat cells above the silicon layer was then counted in scintillation vials. The extracellular [^{3}H]-2-DG present in this upper part of the tubes, which was not internalized in cells, was determined with adipocytes whose transport activity was previously blocked by cytochalasin B at time 0. Though averaging 1%-5% of the radioactivity found in the upper phase, it was subtracted from all assays, as in[17]. Among the slight adaptations that differentiated assays with human fat cells from those with rat or mouse adipocytes was the use of human insulin instead of bovine insulin for rodent preparations and a higher richness in adipocytes: 20 mg lipids/400 µL instead of approximately 15 mg/400 µL.

Lipolytic activity determination

Glucose was present at 5.5 mmol/L in the Krebs-Ringer-based medium used for lipolysis assays, for which 2-DG and pyruvate were omitted, as already reported[15]. As above, tested agents were added to 400 µL of fat cell suspension at the start of a 90-min incubation at 37 °C under gentle shaking. Incubations were stopped on ice. Lipolysis was determined by using glycerol release as an index as already documented, considering that free fatty acid release exhibits parallel variations in our experimental conditions[16].

Statistical analysis

Results are presented as means ± SE of the mean of (*n*) observations. All the statistical analyses for comparisons between parameters used analysis of variance followed by post-hoc Dunnett's multiple comparisons test, analyzed with Prism 6 for Mac OS X (from GraphPad software, Inc). NS means non-significant difference.

RESULTS

Adrenaline and noradrenaline activate hexose transport in human adipocytes without need for vanadium

When incubation medium of human adipocytes was buffered at pH 7.5, no stimulation of basal hexose uptake was obtained with sodium orthovanadate at a final concentration of 100 μmol/L (Figure 1). Even the insulin-stimulated hexose transport, which was approximately three times higher than baseline, was not modified by 100 μmol/L vanadate. However, vanadium addition impressively potentiated the stimulatory effect of 1 mmol/L hydrogen peroxide on hexose uptake into human adipocytes (Figure 1), as already reported for rodent adipocytes[27]. Hydrogen peroxide was tested here as a reference because: (1) It stimulates hexose uptake in human adipocytes [17]; (2) Its action is potentiated by 100 μmol/L vanadate in rodent adipocytes[1]; and (3) It is one of the end-products of amine oxidase activity, regardless of the amine substrate or the type of amine oxidase activated[28].

All these control conditions confirmed our previous observations[15,17] and indicated that the human fat cell preparations were responsive to insulin regarding glucose transport activation. More importantly, the synergism between vanadium and hydrogen peroxide was in line with the characterization of the insulin-like properties of peroxovanadate, the compound generated by the combination of vanadate and hydrogen peroxide[5,29].

Figure 1 also shows that vanadate did not modify the effect of 100 μmol/L benzylamine, which elicited a stimulation that was equivalent to approximately one-third of the maximal insulin stimulation. Similarly, the stimulatory effect of adrenaline and noradrenaline on hexose uptake in human fat cells was not enhanced by vanadate (Figure 1). This was strikingly different from the synergism found between vanadate and amines regarding activation of glucose uptake in rodent adipocytes[1,27].

Together, these first observations indicated that high doses of adrenaline and noradrenaline can acutely activate glucose transport in human fat cells, at least when incubated with the cells at 100 μmol/L for 45 min. The unexpected difference when compared with animal models was that the 'insulin-like' effect of the amines was not enhanced by vanadium in human fat cells. In other words, catecholamines stimulated 2-DG uptake in human adipocytes without the need for vanadium. This capacity to enhance glucose transport deserved further study since it could constitute a novel rationale for increasing glucose consumption in peripheral tissues. We investigated whether other amines could activate 2-DG uptake in human adipocytes, either in a vanadium-dependent or independent manner. To this aim, we compared the responses of rat and human adipocytes.

Comparative study of the glucose transport stimulation by various amines in the absence and the presence of vanadium

Figure 2 shows that the behavior of human adipocytes was clearly different from that of rat adipocytes regarding the synergism between vanadium and amines. The clear potentiation occurring between vanadate and most of the tested amines, already evidenced in rat adipocytes[1], could not be merely extrapolated to human adipocytes. However, this interspecies comparative approach indicated that adrenaline and noradrenaline were the most powerful agents among the fifteen biogenic amines tested in human adipocytes and demonstrated that not any given amine was able to activate glucose uptake at 1 mmol/L. For unknown reasons, the relative rank order of potency for (either cyclic or aliphatic) amines activating hexose uptake was not the same in rat and human adipocytes. Another important finding drawn from this comparison is that the lack of potentiation between vanadium and amines was generalized to all the amines tested on glucose transport in human adipocytes, at least under our experimental conditions.

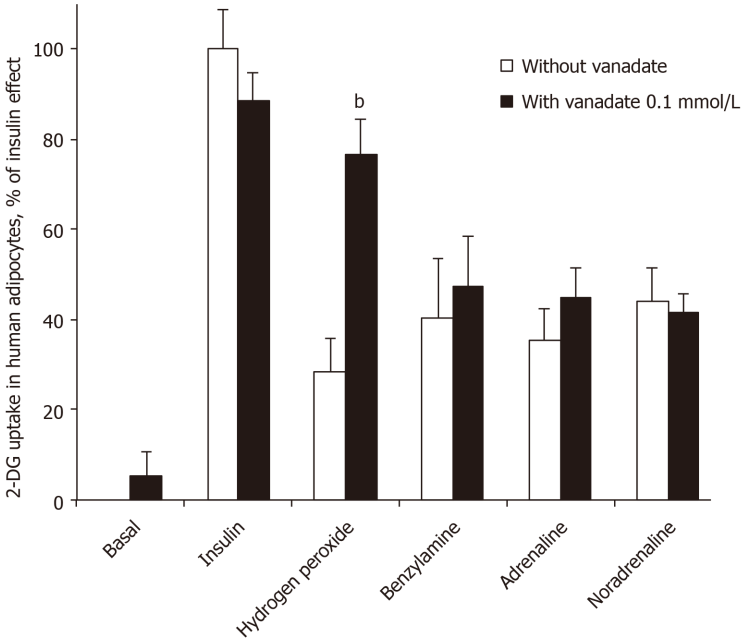

Figure 1 Influence of vanadate on glucose transport in human adipocytes in response to insulin, hydrogen peroxide and naturally occurring amines. Suspensions of human fat cells were preincubated without (white columns) or with 100 μmol/L sodium orthovanadate (dark columns) for 45 min in the presence of the indicated amines at 100 μmol/L or with 100 nmol/L insulin and hydrogen peroxide at 1 mmol/L. [^{3}H]-2-deoxyglucose uptake assay was then immediately performed for 10 min and expressed as the percentage of maximal stimulation by insulin (set at 100%), with baseline set at 0% (the respective levels of which were: 1.29 ± 0.12 and 0.45 ± 0.04 transported nmol 2-deoxyglucose/100 mg cell lipids/10 min). Each column is the mean ± standard error of the mean from 7 to 10 determinations. In all conditions tested, 2-deoxyglucose uptake was significantly different from basal. A significant influence of vanadium when compared to respective control without vanadate was observed at: $^bP < 0.01$. 2-DG: 2-Deoxyglucose.

Comparative study of methylamine-dependent stimulation of glucose transport in rat and human adipocytes

Methylamine, which does not contain any benzene or catechol ring in its chemical structure and is the simplest molecule well-recognized as an SSAO substrate, was then used for further comparison between rat and human adipocytes. The methylamine stimulation of 2-DG uptake, which occurred only in the presence of 100 μmol/L vanadate in rat adipocytes, was entirely abolished by 1 mmol/L semicarbazide, the reference inhibitor of SSAO, while it was partially resistant to the MAO inhibitor pargyline, even when present at 1 mmol/L (Table 1). In human adipocytes, methylamine activated 2-DG uptake without the need for vanadium. The same pattern of inhibition was observed in both species, albeit the glucose uptake activity exhibited lower amplitude in humans (Table 1). Such interspecific difference in the magnitude of the response was rather expected since it has been well established that human adipocytes are less metabolically active than rodent fat cells. This did not prevent drawing of the same conclusion for both models: the combination of pargyline and semicarbazide largely impaired the methylamine activation of hexose transport (Table 1).

Thus, in rats and humans, semicarbazide plus pargyline likely impaired the release of oxidation products during methylamine catabolism by SSAO and/or MAO, and this consequently prevented methylamine to activate 2-DG uptake. It can be postulated that activation of the amine oxidases expressed in adipocytes was supporting the 2-DG uptake activation in response to millimolar doses of methylamine. Hydrogen peroxide, one of the products generated during oxidative deamination was supposed to be involved in this hexose uptake stimulation, according to previous studies in adipocytes[1] or cardiomyocytes[30]. However, this paradigm does not address the different sensitivities of hydrogen peroxide and amines regarding potentiation by vanadium in human fat cells. Additional investigations were therefore required to depict the mechanisms underlying the stimulatory action of catecholamines on the glucose entry into human adipocytes. It was decided to search whether degradation products other than hydrogen peroxide could mediate the catecholamine effect on 2-DG uptake by further comparing rodent and human adipocytes.

Table 1 Methylamine stimulation of hexose uptake in rat and human adipocytes is dependent on semicarbazide-sensitive amine oxidase

	Rat adipocytes with vanadate 0.1 mmol/L	Human adipocytes without vanadium
Incubation condition	2-DG uptake (nmol/100 mg lipids/10 min)	
Control	1.30 ± 0.12	0.49 ± 0.05
Insulin 100 nmol/L	13.08 ± 0.31[e]	1.49 ± 0.19[e]
Methylamine 1 mmol/L	11.27 ± 1.17[e]	0.86 ± 1.3[b]
Met + pargyline 1 mmol/L	6.37 ± 0.88[f]	0.84 ± 0.09
Met + semicarbazide 1 mmol/L	1.32 ± 0.19[f]	0.60 ± 0.08[c]
Met + pargyline + semicarbazide	1.18 ± 0.34[f]	0.59 ± 0.07[d]

Glucose uptake was assayed for 10 min after 45-min incubation with the indicated doses of agents. Mean ± SE of the mean of 8-9 rat and 18 human adipocyte preparations. Difference between insulin or methylamine and control significant at:
[a]$P < 0.05$.
[b]$P < 0.01$.
[e]$P < 0.001$.
Significantly different from methylamine alone at:
[c]$P < 0.05$.
[d]$P < 0.01$.
[f]$P < 0.001$.
2-DG: 2-Deoxyglucose; Met: Methylamine.

Effects of waste metabolites of catecholamine catabolism on glucose transport in mouse and human adipocytes

Since the reaction end-products of benzylamine oxidation by amine oxidases, benzaldehyde and ammonia, have been found to be inactive on glucose transport in human adipocytes[18], they were not further investigated here. By contrast, pyrocatechol and benzoquinone, which can be considered as final metabolites of catecholamine catabolism, have never been tested on glucose transport, at least to our knowledge. Thus, it was investigated how their putative effects could be improved by vanadate. The metabolite pyrocatechol, formed by a benzene core carrying two hydroxyl substituents, was inefficient on glucose transport in both mouse and human adipocytes when tested alone from 1 μmol/L up to 1 mmol/L (Figure 3). Pyrocatechol was also unable to activate hexose uptake in rat adipocytes (not shown). Even when tested with vanadate, pyrocatechol was inefficient in the three species, with only a tendency to generate higher uptake levels in mice, without reaching significance and with largely weaker magnitude than insulin stimulation (Figure 3).

In many biological materials, the oxidation of catechol gives reddish-brown melanoid pigments, derivatives of benzoquinone. We therefore tested benzoquinone on glucose transport. In mouse adipocytes that were highly responsive to insulin, benzoquinone did not notably activate 2-DG uptake, with or without vanadium (Figure 3). Benzoquinone even inhibited basal 2-DG uptake at 1 mmol/L, and a similar pattern was observed in human adipocytes (Figure 3). Apparently, these "waste" products of catecholamine catabolism were not responsible for the mild activation of hexose uptake by high doses of (nor)adrenaline either in rodent[1] or human adipocytes (see Figures 1 and 2). Moreover, benzoquinone was inhibitory at millimolar doses.

An additional investigation was performed with pyrocatechol and benzoquinone on mouse adipocytes and indicated that they were neither able to activate lipolysis as did adrenaline or adrenaline (Figure 4) nor able to impair the lipolytic effect of the catecholamines when tested at 1 μmol/L (not shown). Hence, these waste products cannot be suspected to impair or to support the effects of (nor)adrenaline on glucose entry in human fat cells.

During all these verifications, the sole 2-DG uptake activation demonstrated to depend on amine oxidase activity was that of methylamine. Thus, verifying whether the effects of (nor)adrenaline were sensitive to blockade by pargyline and semicarbazide in human adipocytes remained mandatory.

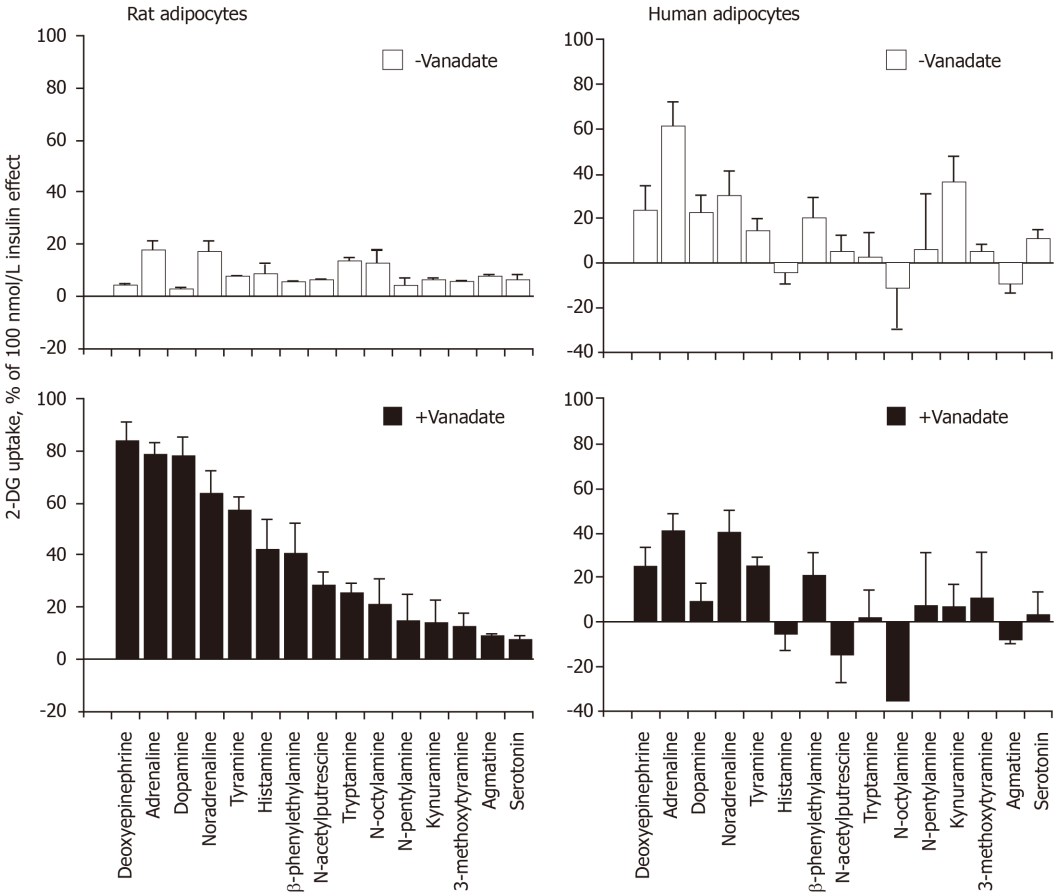

Figure 2 Interspecific differences between rat and human adipocytes in hexose uptake activation by various amines with and without vanadium. Rat (left) or human (right) fat cells were incubated with 1 mmol/L of the indicated amines in the absence (upper panels, open columns) or the presence of 0.1 mmol/L sodium orthovanadate (lower panels, black columns) just before 2-deoxyglucose uptake assays. Hexose uptake was expressed as the percentage of maximal stimulation induced by 100 nmol/L insulin (set at 100%, with basal uptake set at 0). Negative percentages traduced a transport that was lower than baseline. Each column is the mean ± standard error of the mean of 4 to 11 experiments in rat adipocytes and of 3 to 12 preparations of human adipocytes and is presented according to the decreasing rank order of amine-induced stimulation obtained in rat adipocytes when tested at 1 mmol/L + 0.1 mmol/L vanadate, according to our previously published data[1], redrawn here in the left panel with the author's permission. 2-DG: 2-Deoxyglucose.

The activation of hexose uptake in human adipocytes by noradrenaline and adrenaline resists inhibition by pargyline and semicarbazide

The glucose transport in human adipocytes incubated with 100 nmol/L insulin was defined as the maximal activation of 2-DG uptake and set at 100%. Basal and insulin-stimulated 2-DG uptake resisted the blockade by the combination of amine oxidase inhibitors: pargyline + semicarbazide (parg + semi) (Figure 5A). Nevertheless, as above with methylamine, the use of benzylamine and octopamine confirmed that 'classical' amine oxidase substrates were able to activate glucose entry in human fat cells in a manner that was abolished by parg + semi (Figure 5A). Surprisingly, this was not the case for the activation of 2-DG uptake by 0.1 and 1 mmol/L of noradrenaline and adrenaline, which was not impaired by parg + semi, ruling out the contribution of amine oxidase-dependent oxidation (Figure 5B and C). When tested separately, pargyline and semicarbazide were unable, even at 1 mmol/L to inhibit the adrenaline-induced hexose transport (respective 2-DG uptake levels were in nmol/100 mg lipid/10 min: adrenaline, 1.01 ± 0.12; adrenaline + semicarbazide, 1.01 ± 0.17; adrenaline + pargyline , 0.95 ± 0.10; n = 16; NS, not shown).

Being resistant to parg + semi, the stimulatory action of catecholamines on glucose entry in human adipocytes was not dependent on amine oxidase activity as observed in rodents. Albeit we recently ruled out the contribution of β-AR stimulation in the effects of catecholamines plus vanadium on glucose transport in rodent adipocytes[1], it became necessary to explore this putative mechanism in human adipocytes.

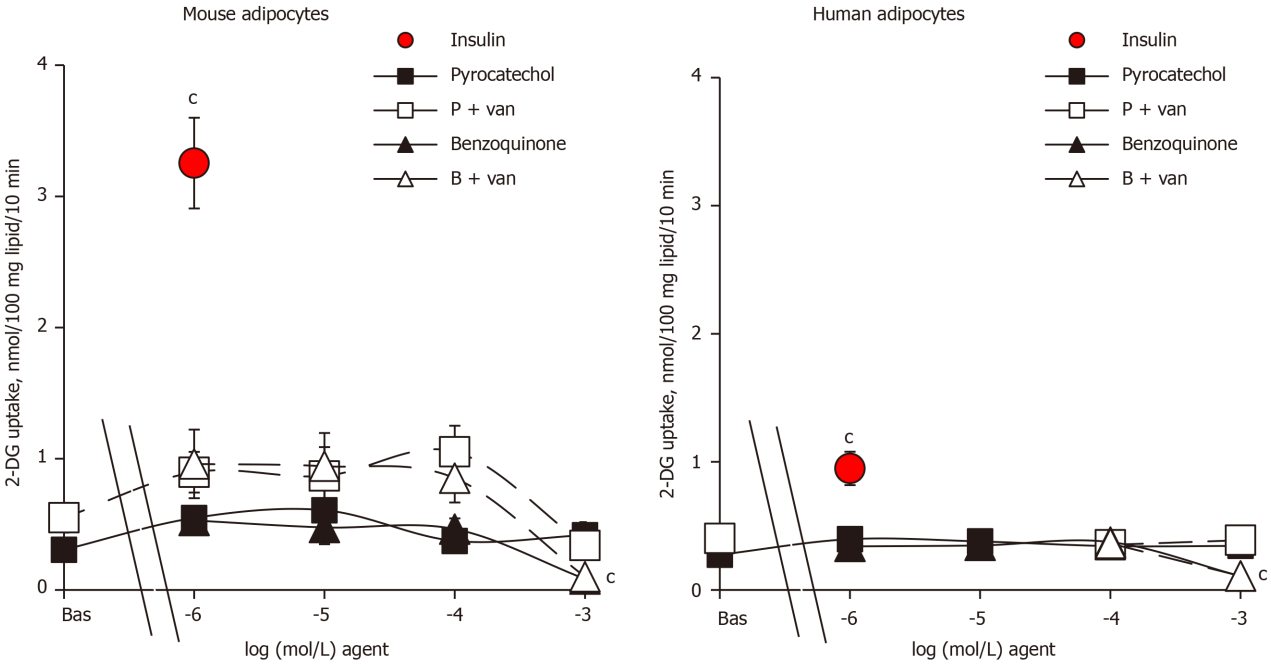

Figure 3 Lack of stimulatory influence of pyrocatechol and benzoquinone on hexose uptake in mouse and human adipocytes. Mouse (left panel) and human (right panel) fat cells were incubated for 45 min with increasing concentrations (from 1 µmol/L to 1 mmol/L, indicated as log of molar concentration) of pyrocatechol (squares) or benzoquinone (triangles) without (closed symbols) or with 100 µmol/L vanadate (open symbols) just before assaying [^{3}H]-2-deoxyglucose uptake for a 10-min period. Basal (without any agent added) and maximal hexose uptake in response to 1 µmol/L insulin (red circle, bovine hormone for mouse adipocytes and human recombinant protein for human adipocytes) are given with the same Y-axis scale for the sake of comparison. Mean ± standard error of the mean of 9 and 7 separate experiments for mouse and human adipocyte preparations containing 15 ± 2 and 20 ± 3 mg lipid/400 µL assay tube, respectively. Difference from basal uptake was significant at: $^cP < 0.001$. 2-DG: 2-Deoxyglucose; B: Benzoquinone; van: Vanadate; P: Pyrocatechol; Bas: Basal.

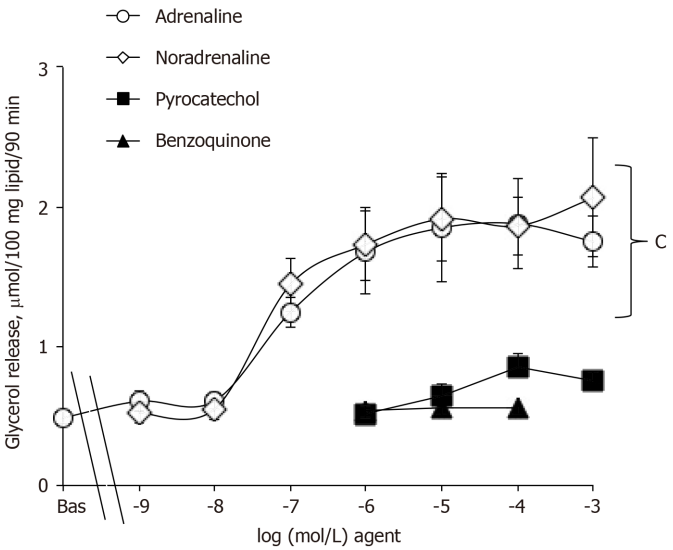

Figure 4 Dose-dependent activation of lipolysis by adrenaline and noradrenaline but not pyrocatechol and benzoquinone in mouse adipocytes. Glycerol release was assessed after 90 min incubation of mouse adipocytes without (basal) or with increasing concentrations of adrenaline (open circles), noradrenaline (open diamonds), pyrocatechol (black squares) or benzoquinone (dark triangles). Each condition is the mean ± standard error of the mean of 5-6 adipocyte preparations. The doses of adrenaline and noradrenaline ranging between 1 µmol/L and 1 mmol/L induced a response different from basal at: $^cP < 0.001$. Bas: Basal.

Is there a direct activation of hexose uptake in human adipocytes by adrenoreceptor agonists or a blockade by adrenergic antagonists?

Figure 6 shows that the glucose transport of human adipocyte preparations that were responsive to human insulin could not be activated notably by any of the five β-adrenergic receptor agonists tested. The α_2-AR agonist UK 14304 (also known as brimonidine) was also inefficient, arguing that α_2-adrenergic activation does not

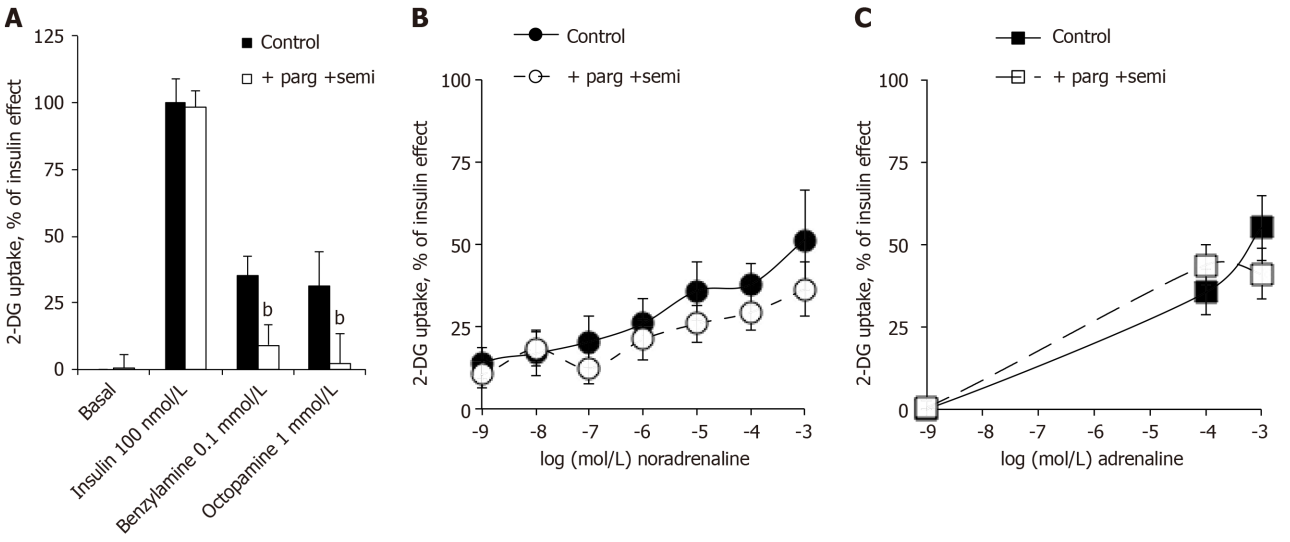

Figure 5 Inhibition by pargyline and semicarbazide of benzylamine and octopamine effects on hexose uptake in human adipocytes but not of noradrenaline and adrenaline effects. Human fat cells were incubated in the presence of the indicated agents without (control, black symbols) and with the combination of 100 µmol/L pargyline plus 1 mmol/L semicarbazide (open symbols) before being subjected to 2-deoxyglucose uptake assay. A: Insulin, benzylamine and octopamine: mean ± standard error of the mean of 7 adipocyte preparations. A significant inhibition when compared to respective control was observed at: $^{b}P < 0.01$. B: Increasing doses of noradrenaline: mean ± standard error of the mean of 13 cases. C: Indicated doses of adrenaline: mean ± standard error of the mean of 17 cases. No significant difference was found between inhibitor and respective control conditions. 2-DG: 2-Deoxyglucose; parg: Pargyline; semi: Semicarbazide.

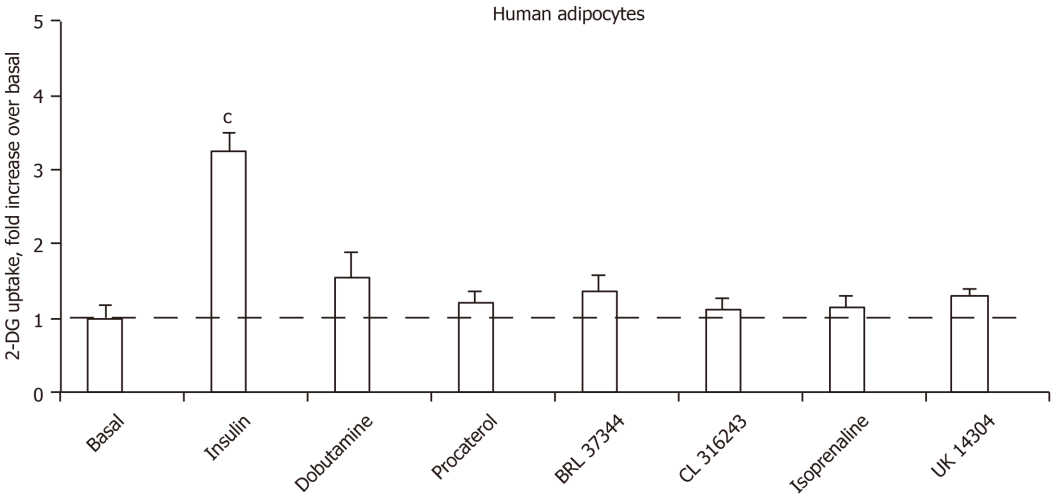

Figure 6 Influence of insulin, β- and α-adrenergic receptor agonists on hexose transport in human adipocytes. 2-Deoxyglucose uptake assay was performed without (basal) and with 100 nmol/L insulin or 1 µmol/L of the indicated adrenergic receptor agonists. Dobutamine: β_1-AR agonist; procaterol: β_2-AR agonist; BRL 37344 and CL 316243: β_3-AR agonists; isoprenaline: pan-agonist of the three subtypes of β-ARs; UK 14304: α_2-AR agonist. Glucose transport was expressed as fold increase relative to basal uptake set at 1.0 (dotted line). Each column is the mean ± standard error of the mean of 9-12 individual adipocyte preparations. Only insulin-induced uptake was significantly different from baseline at: $^{c}P < 0.001$. 2-DG: 2-Deoxyglucose.

enhance glucose uptake (Figure 6). Of note, the tested β_1- and β_2-adrenergic agonists were active at 1 µmol/L on lipolysis activation in human fat cells (or in provoking antilipolytic response in the case of UK 14304), as previously reported in independent studies[31,32].

In additional experiments performed to study the sensitivity to antagonists, RX 821002 was chosen for blocking α_2-ARs, and the pan-antagonist bupranolol for blocking the β-ARs. Again, adrenaline at 100 µmol/L increased the basal 2-DG uptake (basal: 0.34 ± 0.02, adrenaline: 0.66 ± 0.04; $n = 4$; $P < 0.01$), and this was not impaired by 10 µmol/L of each of the antagonists (adrenaline + RX 821002: 0.61 ± 0.06; adrenaline + bupranolol: 0.58 ± 0.04 nmol 2-DG transported/100 mg lipids/10 min; $n = 4$; NS, not shown).

Thus, the use of adrenergic agents could not mimic or block the stimulatory effect of noradrenaline and adrenaline on hexose uptake. At this stage, the role of autoxidation products of the catecholamines was investigated. Alongside adrenochrome and noradrenochrome, the chemistry of catecholamine degradation encompasses numerous ROS, aldehydic molecules and oligomeres implied in neurotoxicity[33]. Rather than testing these highly reactive intermediates, which are rather unstable, it was investigated whether the relatively short-term effect of millimolar doses of adrenaline could be prevented by antioxidant pretreatment. As hydrogen peroxide is active on hexose uptake in adipocytes, it was verified whether its generation was prevented by catalase. Figure 7 shows that catalase impaired the adrenaline-induced stimulation of 2-DG uptake in human adipocytes. The addition of glutathione, expected to limit hydrogen peroxide dismutation by catalase, could not reach complete blockade of adrenaline effect. Lastly, the phosphoinositide 3-kinase inhibitor wortmannin was able at 1 μmol/L to inhibit the effect of adrenaline as well as that of insulin (Figure 7), suggesting that in both cases the activation of hexose uptake was due to a phosphoinositide 3-kinase/protein kinase B-induced glucose carrier recruitment to the cell surface.

DISCUSSION

In a recent study, in view of the powerful stimulating effect of the combination of catecholamines plus sodium orthovanadate on glucose transport in rodent adipocytes, we have proposed that the use of catecholamines might improve the antidiabetic effect of vanadium by reducing its efficient therapeutic doses and by lowering its toxicity[1]. In the present study, we aimed to extrapolate to human adipocytes the description of the insulin-like nature of the synergism between catecholamines and vanadium on glucose utilization. The results of our present human study clearly indicate that adrenaline and noradrenaline activate hexose uptake in human fat cells at doses comprised between 0.1 and 1 mmol/L. In fact, human fat cells respond to catecholamine exposure for 45 min by a stimulation of hexose uptake that represents one-third to one-half of the maximal response to insulin, depending on the individuals. To our knowledge, it is the first time that such a short-term, non-negligible, insulin-like effect of these two naturally occurring catecholamines is observed in human fat cells. However, this stimulation was not further enhanced by the presence of vanadium and never reached the 80%-90% of the maximal insulin-dependent stimulation of glucose uptake, as it was observed in rodents[1]. In other words, the synergism found between (nor)adrenaline and sodium orthovanadate in rat fat cells was not observed in human adipocytes. This interspecific difference, already observed for the sensitivity to decavanadate[5], abruptly ceased our proposal to use catecholamine derivatives in future strategies aimed at improving the benefit/risk ratio of vanadium-based antidiabetic treatments.

However, a potentiation of the mild activation effect of hydrogen peroxide with 0.1 mmol/L vanadate (a dose inefficient on its own to activate 2-DG uptake) occurred in both human and rodent adipocytes (Figure 1 and[1]). It is not the synergism between hydrogen peroxide and vanadium, which generates peroxovanadate, a powerful insulin-mimicking agent on glucose utilization, that was primarily involved in such unexpected interspecific differences. Curiously, in the same experimental conditions, catecholamines were not the sole amines that behaved differently between human and rodents fat cells. The widely recognized SSAO substrates, benzylamine and methylamine, and various other biogenic amines did not exhibit any synergism with vanadium in activating 2-DG uptake in human fat cells, while most of them were potentiated in rodent adipocytes[1,10,34].

Other unexpected differences between rat and human adipocytes did not facilitate a mere extrapolation of our previous findings regarding the synergism between catecholamines and vanadium. For instance, dopamine, which was as stimulatory as adrenaline and noradrenaline in rat adipocytes[1], was not active in human fat cells. Similarly, deoxyepinephrine was much more active on glucose uptake in rats than in humans. Unfortunately, we cannot provide at the moment any explanation for such differences, and this also applies for the rank order of potency for the various amines tested without and with vanadate on hexose uptake, since that found in the rat model is not at all predictive of that found in humans. The catabolism of the biogenic amines and the fates of the vanadate/vanadyl forms of the metal ion are probably different in the two species, as it is also the case for hydrogen peroxide generation/catabolism.

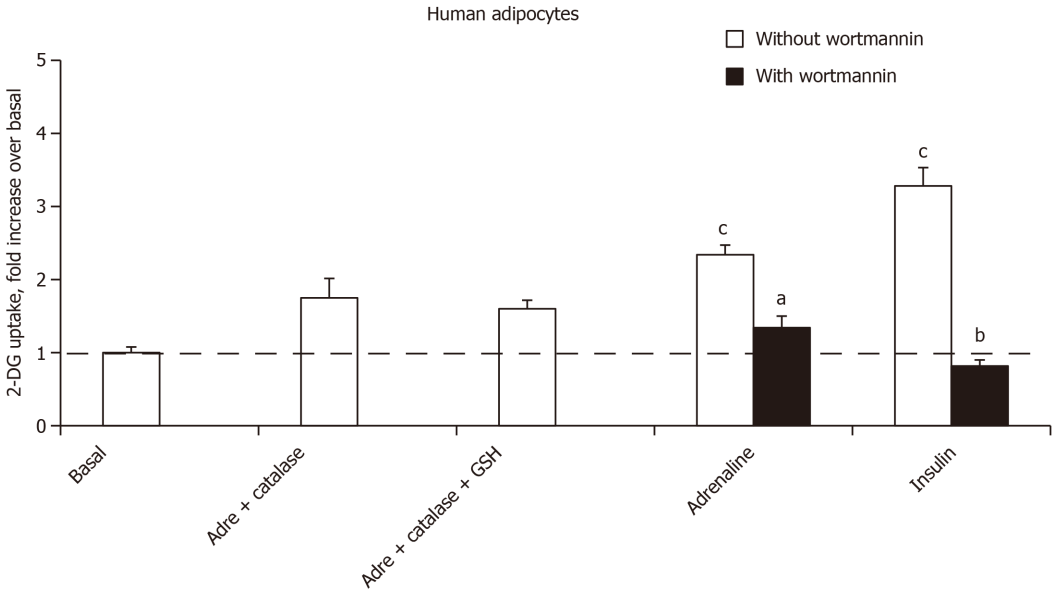

Figure 7 Inhibition of adrenaline-induced hexose uptake in human adipocytes by catalase, glutathione and wortmannin. The pretreatment of adipocytes by catalase alone (5000 IU/mL) or in combination with 1 mmol/L glutathione was started 10 min before the addition of 1 mmol/L adrenaline, which was incubated for 45 min before performing 2-deoxyglucose uptake assay for 10 min. The mean ± standard error of the mean of 4 to 6 separate experiments is given as fold increase above basal 2-deoxyglucose uptake. A significant difference from basal was observed at: $^{c}P < 0.001$; Different from 1 mmol/L adrenaline alone at: $^{a}P < 0.05$, or from 100 nmol/L insulin alone at: $^{b}P < 0.001$. 2-DG: 2-Deoxyglucose; adre: Adrenaline.

Nonetheless, several common features were observed in this comparative approach: first, no significant effect of serotonin was evidenced in both species; then, the same dose of vanadate that was inefficient on its own on basal or insulin-stimulated hexose uptake, *i.e.*, 0.1 mmol/L, potentiated the hydrogen peroxide effect in both species. Other points of resemblance between rodent and human adipocytes are discussed below, but it must be kept in mind that the essential difference between the two models is that rat fat cells are definitely more metabolic active than the human ones, as attested by the absolute values of maximal hexose transport in response to insulin (see Figure 3 and Table 1).

Although impressed by the distinct intrinsic activity and vanadium sensitivity of the fifteen amines tested, we further attempted to decipher the mechanisms of action implied in the two most active on glucose uptake in human adipocytes: adrenaline and noradrenaline. As with rodent adipocytes, adrenaline and noradrenaline behaved differently from typical SSAO substrates (benzylamine, methylamine): only the latter were not able to activate glucose uptake in the presence of semicarbazide, alone or combined with the monoamine oxidase inhibitor pargyline. In this aspect, human adipocytes resemble the rat ones. Nevertheless, the lack of inhibition of (nor)-adrenaline effect by the combination parg + semi is puzzling since catecholamines are well-known substrates of MAO, abundant in adipocytes[27].

We used the combination of MAO and SSAO inhibitors in the present study since both MAO and SSAO substrates are able to mimic insulin-like effects in adipocyte models[27], and since methylamine is a product of adrenaline oxidation by MAO together with hydrogen peroxide. In turn, methylamine is a substrate for SSAO, also generating hydrogen peroxide[35]. This postulated two-step process could explain why adrenaline was the most powerful among the amines tested in stimulating glucose uptake in human adipocytes. But it cannot explain why the adrenaline effect was so weakly impaired by parg + semi combination, capable to block the methylamine-induced glucose transport. Moreover, semicarbazide, although inactive on MAO, inhibits the activity of the enzymes encoded by the *AOC1* and *AOC3* genes (diamine oxidase and SSAO) and other related copper containing amine oxidases[36]. Despite its similitude with our previous observations in rodent adipocytes[1], the resistance to the blockade by the parg + semi combination remains a characteristic of the effects of adrenaline and noradrenaline that is not totally elucidated. Rather than testing the presence of other putative amine oxidases that could be implied in the oxidation of adrenaline and noradrenaline, we explored possible transduction signals other than the amine oxidase-mediated pathway.

The absence of plateau in the dose-response curve to noradrenaline activation of 2-DG uptake in human fat cells was somewhat indicative that the mechanism involved is not mediated by a single receptor activation. Indeed, the linear increase of uptake in response to noradrenaline from 1 nmol/L to 1 mmol/L found in human adipocytes clearly contrasted with the typical sigmoid curve seen in mouse adipocytes when the adrenergic stimulation of glycerol release was determined. Only the latter response corresponds to a classical activation of the lipolytic cascade, implying an amplification system with successive activation of β-AR/Gs protein/adenylyl cyclase/protein kinase A/lipases (compare Figures 4 and 5). In keeping with this, none of the various adrenergic agonists tested was able to activate 2-DG uptake in human adipocytes, and the effect of adrenaline was insensitive to the α_2- and β-AR antagonists used. These results were in perfect agreement with our recent report showing that β-AR or α_2-adrenergic receptor stimulation was not involved in the stimulation by catecholamines plus vanadium of glucose transport in rodent adipocytes[1]. Our pharmacological approach still leaves open a putative mediation of the glucose transport stimulation by α_1-AR activation, as proposed in a clinical study based on the effect of noradrenaline and the α_1-AR agonist norfenefrine during microdialysis experiments in obese patients [37]. When keeping in mind that neither α_1-AR agonist nor α_1-AR antagonist modified 2-DG uptake in rat fat cells[1], such α_1-AR contribution does not appear plausible and cannot be the sole mechanism supporting the glucose uptake stimulation by 100 μmol/L noradrenaline or adrenaline. Even the stimulation of glucose uptake in rat cardiomyocytes by the recognized α_1-AR agonist phenylephrine has been reported to be biphasic: mediated partly by calcium release and by hydrogen peroxide[38].

It was then the products of catecholamine autoxidation that were suspected to produce activation of hexose uptake in view of: (1) The lack of classical sigmoid shape of the dose-response curve to adrenaline; (2) The resistance to amine oxidase inhibitors, although some hydrazine derivatives have been proven to limit the lipid oxidation by reactive carbonyl compounds[39]; and (3) The impairment caused by antioxidants on the activation by adrenaline + vanadate in rodent adipocytes[1].

The autoxidation of (nor)adrenaline, which generates (nor)adrenochrome and known to be increased by metal ions, can be delayed by EDTA or pH acidification. These two conditions have not been tested in the present study since they directly interfere with glucose transport activity. Although we did not assess whether the presence of vanadium was increasing adrenochrome generation in adipocyte preparations, we did not note any dark coloration in the incubation tubes under any condition. In addition, it must be repeated here that the addition of vanadium to adipocyte incubation medium did not increase the catecholamine-stimulated hexose uptake in human fat cells. However, we were aware that sodium vanadate can elicit pH alkalinization and thereby hexose uptake stimulation. For this reason, we prevented any pH elevation by 0.1 mmol/L vanadate owing to the strongly buffered incubation medium we used. Thus, the putative contribution of adrenochrome in the observed effects is not dealing with the lack of potentiation of adrenaline-induced uptake by vanadate since it has been reported that vanadate enhances the *in vitro* formation of adrenochrome from epinephrine, alongside a reduction of antioxidative defenses, a property that might be linked to vanadate toxic effects in various cell types [40,41]. The fact that the adrenaline stimulation of glucose transport was limited by catalase treatment is another element for discarding the involvement of adrenochrome. Nevertheless, its putative role remains to be definitely ruled out.

One of the limitations in our approach is that we cannot depict the signal transduction elicited by catecholamines when partially mimicking the insulin stimulation of glucose transport. Although we tested two among the numerous metabolites of catecholamines, we did not pay attention to the transient and highly reactive aldehydic molecules generated during either autoxidation or during catabolism by MAO and catechol-O-methyltransferase[33]. Moreover, we did not determine whether there was an appearance of the quinones that are produced during the degradation of (nor)adrenaline into (nor)adrenochrome[42]. However, in accordance with the cytotoxicity of these products, the millimolar dose of benzoquinone has been found to abolish transport activity in adipocytes. While various quinones probably occurred with dopamine also, they did not elicit a detectable effect on 2-DG uptake. Thus, the quinone-based toxic metabolites do not seem to support the catecholamine effect.

It cannot be excluded that others of the numerous metabolites of catecholamine degradation are involved in the *in vitro* effect we detected, but the participation of hydrogen peroxide, endowed with insulin-like effects, could not be clearly evidenced in our experiments, excepted by catalase treatment. Catalase and glutathione were used since they have been shown to protect neuroblastoma cells against the

cytotoxicity of dopamine, due to oxidative stress by generating excessive ROS *via* MAO-catalyzed oxidative deamination and *via* autoxidation[43]. On the contrary, ascorbic acid has been reported to be unable to prevent the autoxidation of catecholamines that occurs readily in the oxygen-saturated incubation media of *in vitro* experiments[44,45]. At last, the inhibition by wortmannin allowed postulating that high doses of catecholamines were activating the recruitment of glucose transporters at the surface of human fat cells.

Finally, noradrenaline and adrenaline are vasoconstrictor agents that stimulate cardiac inotropism, strongly elevate blood pressure as well as increase blood glucose in order to better respond to stress conditions by a behavior well-known from invertebrates to vertebrates as the "fight or flight" response. It is not so astonishing to observe that at high doses these catecholamines are able to activate the glucose utilization in cells in order to facilitate energy consumption. Although the adipocytes are specialized for releasing their lipid stores when the organism requires energy supply, they have to increase glucose uptake/consumption at the same time to perform fatty acid re-esterification to avoid excessive lipolysis. The simultaneous activation of lipolysis and the enhancement of other metabolic pathways such as lipogenesis of fatty acid oxidation is therefore physiologically relevant under adrenergic activation and seems to occur in both animal and human adipocytes. It is the potentiation of the somewhat "insulin-mimicking" properties of catecholamines that does not occur with vanadium in human adipocytes. This does not preclude the interest of the current improvements of the antidiabetic therapeutic applications of vanadium[9,46,47] but seriously limits the relevance of the observations made on rat adipocytes[1,27,48] regarding the promising insulin mimicry of vanadium compounds.

CONCLUSION

This preclinical study describes *in vitro* the activation of hexose uptake in human adipocytes by high doses of catecholamines. It also demonstrates that this insulin mimicry has no interest for improving the benefit/risk ratio of vanadium-based antidiabetic complexes since there is no synergism between catecholamines and vanadate regarding glucose uptake in isolated human adipocytes. Moreover the puzzling effect of catecholamines is not entirely mediated by adrenoreceptor stimulation or by MAO- and SSAO-dependent amine oxidation. As lower doses of catecholamines are recognized to rise blood pressure and blood glucose *in vivo*, no therapeutic use of the present observations can be postulated at the present time.

ARTICLE HIGHLIGHTS

Research background

We have recently reported a synergism between vanadium and catecholamines that generates a powerful activation of glucose transport in rodent adipose cells. Since the combination vanadium/adrenaline or vanadium/noradrenaline mimicked insulin activation of glucose handling in a manner depending on the production of reactive oxygen species, we proposed that further research on vanadate/catecholamine complexes could develop novel, less toxic antidiabetic therapeutic approaches for vanadium compounds.

Research motivation

To extrapolate to humans the potential antihyperglycemic properties of the vanadate/catecholamine combination found in animal models, we aimed to verify whether several amines, including adrenaline and noradrenaline, were able together with vanadate to reproduce the insulin-induced stimulation of glucose transport into human adipocytes.

Research objectives

To evaluate the impact of various biogenic amines, including the well-known catecholamines, adrenaline and noradrenaline, without and with vanadium, on glucose transport in human adipose cells.

Research methods

Preparations of freshly isolated human adipocytes, obtained from patients undergoing plastic surgery, were subjected to a pharmacological exploration of glucose transport owing to short-term uptake assays performed with the non-metabolizable radiolabeled analogue 2-deoxyglucose. An interspecies approach compared the responses of rat, mouse and human adipocytes subjected to similar stimuli.

Research results

In human adipose cells, the stimulation of glucose transport by insulin increased by two-to three times the basal uptake. Neither basal nor insulin-stimulated glucose transport was altered by 100 μmol/L sodium orthovanadate, which clearly potentiated the mild stimulatory action of hydrogen peroxide. Among fifteen biogenic amines tested, adrenaline and noradrenaline were the most efficient in activating 2-deoxyglucose uptake. The stimulation occurred within 0.01-1 mmol/L dose range and was not enhanced with vanadium. Although known to be monoamine oxidase substrates, the stimulation induced by adrenaline and noradrenaline resisted the blockade by amine oxidase inhibitors, as previously found for rodent adipocytes. The tested α- and β-adrenergic agonists did not stimulate glucose uptake in human adipocytes, and the effects of catecholamines were not inhibited by adrenergic antagonists. Benzoquinone and pyrocatechol, two of the various metabolites of catecholamine catabolism were ineffective. Only catalase, together with the antioxidant glutathione, impaired the adrenaline stimulated glucose uptake.

Research conclusions

The powerful synergism of vanadium/catecholamines previously reported on rodent adipocytes was not detectable in human fat cells. Nevertheless, adrenaline and noradrenaline were more stimulatory of hexose uptake than equivalent doses of vanadate, in a manner that was independent from adrenoceptor stimulation or amine oxidase activity.

Research perspectives

If future studies demonstrate an improvement of the antidiabetic properties of vanadium complexes *via* their combination with catecholamines, such improvement will likely not be the result of a synergistic effect on the glucose handling by fat cells.

ACKNOWLEDGEMENTS

We thank the staff of Plastic Surgery Department of Rangueil Hospital (Toulouse, France) and the animal unit CREFRE, more especially its Rangueil satellite for housing rodents. The authors also thank Danielle Prévot for assistance, Xavier Testar (Univ Barcelona) for his grasp of knowledge about vanadium and Anne Bouloumié (Toulouse) for helpful discussions.

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World Journal of Diabetes

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World J Diabetes 2022 January 15; 13(1): 54-64

DOI: 10.4239/wjd.v13.i1.54

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Role of nutritional ketosis in the improvement of metabolic parameters following bariatric surgery

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Institutional review board statement: The protocol was approved by the Institutional Ethics Committee (Keto-BMS study).

Informed consent statement: All study participants, or their legal guardian, provided informed

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Abstract

BACKGROUND

Ketone bodies (KB) might act as potential metabolic modulators besides serving as energy substrates. Bariatric metabolic surgery (BMS) offers a unique opportunity to study nutritional ketosis, as acute postoperative caloric restriction leads to increased lipolysis and circulating free fatty acids.

AIM

To characterize the relationship between KB production, weight loss (WL) and metabolic changes following BMS.

METHODS

For this retrospective study we enrolled male and female subjects aged 18-65 years who underwent BMS at a single Institution. Data on demographics, anthropometrics, body composition, laboratory values and urinary KB were collected.

RESULTS

Thirty-nine patients had data available for analyses [74.4% women, mean age 46.5 ± 9.0 years, median body mass index 41.0 (38.5; 45.4) kg/m^2, fat mass 45.2% ±

written consent prior to study enrollment.

Conflict-of-interest statement: The authors have none to declare.

Data sharing statement: No additional data are available.

Country/Territory of origin: Italy

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: May 8, 2021
Peer-review started: May 8, 2021
First decision: June 16, 2021
Revised: June 29, 2021
Accepted: January 5, 2022
Article in press: January 5, 2022
Published online: January 15, 2022

P-Reviewer: Koritala T
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

6.2%, 23.1% had diabetes, 43.6% arterial hypertension and 74.4% liver steatosis]. At 46.0 ± 13.6 d post-surgery, subjects had lost 12.0% ± 3.6% of pre-operative weight. Sixty-nine percent developed ketonuria. Those with nutritional ketosis were significantly younger [42.9 (37.6; 50.7) years *vs* 51.9 (48.3; 59.9) years, P = 0.018], and had significantly lower fasting glucose [89.5 (82.5; 96.3) mg/dL *vs* 96.0 (91.0; 105.3) mg/dL, P = 0.025] and triglyceride levels [108.0 (84.5; 152.5) mg/dL *vs* 152.0 (124.0; 186.0) mg/dL, P = 0.045] *vs* those with ketosis. At 6 mo, percent WL was greater in those with postoperative ketosis (-27.5% ± 5.1% *vs* 23.8% ± 4.3%, P = 0.035). Urinary KBs correlated with percent WL at 6 and 12 mo. Other metabolic changes were similar.

CONCLUSION
Our data support the hypothesis that subjects with worse metabolic status have reduced ketogenic capacity and, thereby, exhibit a lower WL following BMS.

Key Words: Obesity; Ketone bodies; Bariatric surgery; Weight loss; Glucose metabolism; Lipid metabolism

Core Tip: Ketone bodies might act as potential metabolic modulators besides serving as energy substrates. Acute postoperative caloric and carbohydrate restriction after bariatric metabolic surgery (BMS) leads to increased lipolysis, inducing ketogenesis. We report that the majority, but not all patients undergoing BMS, develop nutritional ketosis. Patients with nutritional ketosis had significantly lower baseline fasting glucose and triglyceride levels *vs* those without ketonuria. Weight loss was greater in those with postoperative ketonuria, and urinary ketones positively correlated with percent weight loss. These observations suggest that subjects with worse gluco-metabolic status have reduced ketogenic capacity, which might blunt the metabolic response to BMS.

Citation: Pindozzi F, Socci C, Bissolati M, Marchi M, Devecchi E, Saibene A, Conte C. Role of nutritional ketosis in the improvement of metabolic parameters following bariatric surgery. *World J Diabetes* 2022; 13(1): 54-64
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/54.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.54

INTRODUCTION

Ketogenesis primarily occurs in the liver at rates proportional to total fat oxidation under conditions of reduced glucose availability such as fasting or very low-calorie ketogenic diets (VLCKDs). In brief, under these conditions, lipolysis-derived free fatty acids (FFAs) undergo beta-oxidation and are broken down into acetyl CoA, which is then converted to ketone bodies (KB), namely acetone, acetoacetate (AcAc), and beta-hydroxy butyrate (BHB), in the mitochondrial matrix of hepatocytes. KBs, namely BHB, AcAc and acetone, transfer lipid-derived energy from the liver, which cannot use them as a fuel, to extrahepatic organs (*e.g.*, central nervous system, heart, skeletal muscle, kidney), serving as an energy substrate alternative to glucose[1]. Over the past few years, the interest in KBs and nutritional ketosis has progressively increased, largely due to the discovery that, besides serving as energy substrates, KBs may also exert favourable metabolic effects[2,3], serving as metabolic regulators and signalling molecules. In particular, BHB exerts antioxidant and anti-inflammatory effects, may affect epigenetics by inhibiting histone deacetylation, suppresses the activity of the sympathetic nervous system and reduces lipolysis and, through unknown mechanisms, to play a role in appetite suppression[4,5]. In healthy individuals, even small increases in KB levels were shown to lower glucose and circulating FFA independent of insulin and glucagon[6], and to attenuate the glycaemic response to an oral glucose tolerance test by increasing insulin sensitivity[7], suggesting a direct metabolic effect of KBs. Bariatric metabolic surgery (BMS) offers a unique opportunity to study

nutritional ketosis, avoiding the complexity of a nutritional intervention such as VLCKD that would need greater effort from patients and also greater costs[8]. Similar to VLCKDs, BMS involves a marked energy deficit that results in massive mobilization of FFAs from adipose tissue and therefore ketogenesis[9,10]. The role of BMS in achieving sustained weight loss (WL), improving obesity-related comorbidities and reducing mortality is well established[11]. However, not all subjects respond to a similar extent[12], those with cardiometabolic abnormalities such as diabetes (especially when long-standing or poorly controlled) and arterial hypertension exhibiting poorer WL after surgery[13,14]. To the best of our knowledge, no studies have assessed the relationship between ketogenic capacity, as reflected by KB production in response to marked calorie restriction, and WL after BMS. We hypothesized that subjects with reduced ketogenic capacity are poorer responders to BMS in terms of WL 6 mo surgery.

MATERIALS AND METHODS

Study design

This was an observational, retrospective, single-centre study part of the KETO-BMS study. Male and female subjects aged 18-65 years who underwent laparoscopic sleeve gastrectomy at San Raffaele Scientific Institute from May 2016 to November 2018 and had urinary KB measured within two months of surgery and a follow-up of at least 6 mo were included. The protocol was approved by the Institutional Ethics Committee, and all patients provided informed consent. All patients underwent routine assessments prior to BMS, including medical history, physical examination, measurement of anthropometrics [height (cm), weight (kg) and body mass index (BMI), calculated as the ratio between the weight and the height squared, waist circumference (WC)], body composition (measured by electric bio-impedance in the fasting state using a BIA AKERN device and the software Bodygram PLUS software, Akern, Montachiello, Italy). Metabolic parameters including fasting plasma glucose (FPG), total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were collected. During the first 8 wk after surgery, patients meet with a registered dietitian and subsequently with a staff physician for nutritional assessment and guidance. As per institutional protocols, during this time frame patients move from clear liquids to pureed foods, progressively increasing to approximately 750-900 kcal daily, depending on protein requirements (up to 1.5g/kg IBW). After the first 8 wk, patients move to solid foods and gradually increase the daily energy intake. Assessments are scheduled every 3-6 mo for the first 12 mo, and annually thereafter. Follow-up outpatient visits include medical history review, physical examination, measurement of anthropometrics, and laboratory assessments as per current recommendations[15].

KB production

KB production was assessed by the presence of acetoacetic acid in urine using an automated dipstick urinalysis (Aution MAX and Aution Sticks, Menarini Diagnostics, Florence, Italy). This is a semiquantitative method that detects urinary acetoacetic acid at concentrations ranging from 5 mg/dL to 150 mg/dL.

Statistical analysis

Descriptive statistics were obtained for all study variables. Normality was assessed with the Shapiro-Wilk test. Continuous variables were expressed as mean ± SD or median (25^{th}-75^{th} percentile), depending on data distribution. Categorical variables were summarised as counts and percentages. Missing data were not imputed. The *t*-test, Welch *t*-test, or Mann-Whitney *U*-test were used for between-group comparisons, depending on variable distribution. The Fisher's exact test was used to assess the association between categorical variables and KB production.

Our primary objective was to examine the relationship between KB production and WL at 6 mo after BMS. One-way analyses of covariance were conducted to examine the effect of sex and pre-operative cardiometabolic conditions [diabetes mellitus (DM), hypertension, dyslipidaemia] on WL at 6 mo, with age included as a covariate. Bivariate correlation analyses were performed to examine the relationship of WL at 6 mo with pre-operative BMI, fat mass, FPG, total cholesterol, triglycerides and post-operative urinary KBs. Relevant variables that were significantly correlated were included in a hierarchical multiple-regression analysis, while controlling for sex, age and BMI. All variables were screened for violations of the assumptions relevant to

each of the statistical analysis performed. Statistical significance was set at $P < 0.05$. Statistical analysis was conducted using IBM SPSS Statistics (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, United States: IBM Corp.).

RESULTS

Patient population

A total of 39 patients were included in the analysis. Patient characteristics are depicted in Table 1. Patients were middled-aged, mostly females. Metabolic-associated fatty liver disease was the most prevalent obesity complication, followed by dyslipidaemia, hypertension and DM.

KB production

Most patients (69.2%) developed ketosis after a mean of 46.0 ± 13.6 d from surgery. Time from surgery was similar between those who did or did not develop ketosis (44.6 ± 15.0 d *vs* 49.0 ± 9.5 d, respectively; $P = 0.351$). Patients with ketosis were significantly younger and had significantly lower pre-operative FPG and triglyceride levels, but greater LDL cholesterol (Table 2). Urinary KBs were inversely correlated with age (Spearman's rho -0.519, $P = 0.001$), FPG (Spearman's rho -0.366, $P = 0.024$), and positively correlated with LDL cholesterol (Spearman's rho 0.426, $P = 0.011$). There was no correlation between urinary KBs and BMI ($P = 0.936$), WC ($P = 0.619$), percent fat mass ($P = 0.768$), total cholesterol ($P = 0.368$), HDL cholesterol ($P = 0.618$) or triglycerides ($P = 0.095$).

WL after surgery

Mean WL at 6 mo was 26.4% ± 5.1% of pre-operative weight in the whole group. WL at 6 mo was significantly greater in patients who had developed post-operative ketosis ($P = 0.035$; Figure 1). Time of assessment was similar between those who did or did not develop ketosis (6.1 ± 0.9 mo *vs* 6.1 ± 0.5 mo, respectively; $P = 0.931$). In 35 patients who had available data at 12 mo (89.7% of the total, 24 and 11 in the group with and without ketosis, respectively), WL also tended to be greater in those with post-operative ketosis ($P = 0.067$, Figure 1). Time of assessment was similar between those who did or did not develop ketosis (11.9 ± 0.9 mo *vs* 12.1 ± 1.2 mo, respectively; $P = 0.590$).

Urinary KB (Spearman's rho 0.398, $P = 0.012$) significantly correlated with WL at 6 mo, whereas age ($P = 0.290$), BMI ($P = 0.056$), fat mass ($P = 0.735$), FPG ($P = 0.680$), total cholesterol ($P = 0.508$) and triglycerides ($P = 0.976$) did not. After adjustment for age, there was a statistically significant difference in WL at 6 mo between males and females, $F(1, 36) = 5.221$, $P = 0.028$, partial $\eta^2 = 0.127$. There was no statistically significant difference between patients with or without DM, hypertension, or dyslipidaemia, therefore these variables were not included in the regression model. At hierarchical multiple regression, urinary KBs and male sex emerged as significant predictors of WL at six months. The full model statistically significantly predicted WL at 6 mo, $R^2 = 0.31$, $F(4, 34) = 3.76$, $P = 0.012$ (Table 3). Urinary KBs also correlated with WL at 12 mo (Spearman's rho 0.356, $P = 0.036$).

Laboratory variables at 6 mo were available for a subgroup of patients. No statistically significant differences in percent change from pre-surgery to 6 mo were detected between groups (Figure 2), although patients who had developed nutritional ketosis tended to have a greater percent increase in HDL cholesterol and greater percent reductions in total and LDL-cholesterol, whereas those who did not develop ketosis tended to have a greater reduction in triglycerides.

DISCUSSION

In this analysis of patients who underwent BMS, we found that KB production during marked calorie restriction after surgery predicted WL at 6 mo. Patients who developed nutritional ketosis had greater WL at 6 mo and tended to have a greater WL at 12 mo after surgery, as compared with those who did not develop nutritional ketosis.

Little information is available on KB production after BMS. Crujeiras *et al*[9] reported that patients who underwent BMS developed mild ketosis at one month after surgery. Thereafter, KBs decreased and returned to pre-operative levels at 3 mo. The association between nutritional ketosis and WL was not explored in that study, which

Table 1 Pre-operative patient characteristics

	All 39	Missing
Age, yr	46.5 ± 9.0	-
Male, *n* (%)	10 (25.6)	-
Hypertension, *n* (%)	17 (43.6)	-
Diabetes mellitus, *n* (%)	9 (23.1)	-
Dyslipidaemia, *n* (%)	22 (56.4)	-
MAFLD	29 (74.4)	-
Waist circumference (cm)		2
Males	129.7 ± 6.2	
Females	114.1 ± 13.3	
BMI, kg/m^2	41.0 (38.5; 45.4)	-
Fat mass (%)	45.2 ± 6.2	5
Plasma glucose (mg/dL)	91.0 (84.0; 98.3)	1
Total cholesterol (mg/dL)	193.1 ± 29.6	3
HDL cholesterol (mg/dL)	48.0 (42.0; 58.0)	4
LDL cholesterol (mg/dL)	115.1 ± 28.0	4
Triglycerides (mg/dL)	118.5 (102.3; 159.3)	3

MAFLD: Metabolic-associated fatty liver disease; BMI: Body mass index; HbA1c: Glycated haemoglobin; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

had a different aim. There may be different explanations for the association between post-operative nutritional ketosis and the greater WL at 6 mo observed in our study. It has been reported that conditions of altered glucose metabolism such as type 2 DM negatively impact WL after BMS[16,17]. Ketogenic capacity might be a proxy of glucometabolic health. Previous studies suggested that ketogenic capacity is impaired in women with obesity as compared to normal-weight controls[18], in the pathogenesis of non-alcoholic liver disease and progression to non-alcoholic steatohepatitis, and even hepatocellular carcinoma[19-21]. Furthermore, studies in mice indicate that impaired ketogenesis may play a role in fatty liver injury and dysregulated glucose homeostasis[22-24]. Patients who did not develop nutritional ketosis in our cohort had significantly higher FPG and triglycerides, indicating worse glucometabolic status. Impaired ketogenesis may be responsible for a diminished extraction of available fat, altered acetyl-CoA balance in mitochondria, and diversion of non-disposed FFAs to other metabolic pathways, possibly including lipogenesis [23]. Conversely, better WL and metabolic responses to BMS in patients with adequate ketogenic capacity might be due to efficient clearance of excess FFAs released from adipose tissue. It has been known for more than 40 years that KBs may have roles beyond serving as energy substrates[25]. Specifically, BHB appears to exert antioxidant and anti-inflammatory effects, to inhibit histone deacetylation and to play a role in appetite suppression[4,5]. In healthy individuals, even small increases in circulating KBs were shown to reduce glucose and triglyceride levels, and to hamper the glycaemic response to an oral glucose load by increasing insulin sensitivity[6,7]. At the time of KB assessment, WL was similar between patients with or without ketosis. However, it is tempting to speculate that exposure to mild ketosis led to an improvement of mitochondrial bioenergetics and metabolic health[3,26,27], which in turn resulted in improved subsequent WL. Despite having significantly higher LDL cholesterol prior to surgery, patients who developed nutritional ketosis exhibited a numerically greater reduction in LDL at 6 mo as compared with patients who did not develop nutritional ketosis (Figure 2). During ketogenesis, acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by mitochondrial HMG-CoA synthase, an enzyme that is also involved in cholesterol synthesis[28]. It is possible that, in conditions of low glucose and high FFA availability, an increase in ketogenesis results in lower rates of *de novo* cholesterol synthesis.

Table 2 Comparison of pre-operative characteristics between subjects who developed (patients with post-operative ketosis) or did not develop (patients without post-operative ketosis) ketosis after surgery

	KB+ (*n* = 27)	KB- (*n* = 12)	*P* value
Age, yr	42.9 (37.6; 50.7)	51.9 (48.3; 59.9)	**0.018**
Female, *n* (%)	20 (74.1)	9 (75.0)	1.000
Hypertension, *n* (%)	11 (40.7)	6 (50.0)	0.730
Diabetes mellitus, *n* (%)	4 (14.8)	5 (41.7)	0.102
Dyslipidaemia, *n* (%)	14 (51.9)	8 (66.7)	0.494
MAFLD	20 (74.1)	9 (75.0)	0.683
Waist circumference[1] (cm)	119.3 ± 13.5	115.3 ± 14.0	0.421
BMI, kg/m^2	41.0 (38.7; 45.4)	40.1 (35.9; 45.6)	0.663
Fat mass (%)	45.6 ± 6.2	44.2 ± 6.1	0.552
Plasma glucose (mg/dL)	89.5 (82.5; 96.3)	96.0 (91.0; 105.3)	**0.025**
HbA1c (mmol/mol)	37.0 (35.8; 41.0)	38.5 (36.0; 46.3)	0.305
Total cholesterol (mg/dL)	197.1 ± 25.8	183.9 ± 36.4	0.222
HDL cholesterol (mg/dL)	48.0 (42.5; 53.0)	49.0 (39.5; 62.0)	0.843
LDL cholesterol (mg/dL)	121.0 ± 23.5	100.2 ± 33.9	**0.045**
Triglycerides (mg/dL)	108.0 (84.5; 152.5)	152.0 (124.0; 186.0)	**0.020**

[1]Pooled data for males and females, as there were only 2 males in the patients without post-operative ketosis group.
MAFLD: Metabolic-associated fatty liver disease; BMI: Body mass index; HbA1c: Glycated haemoglobin; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; KB: Ketone bodies; WL: Weight loss.

Table 3 Hierarchical regression analysis for weight loss at 6 mo

	Weight loss at 6 mo					
	Model 1		Model 2		Model 3	
Variable	B	β	B	β	B	β
Constant	30.300[b]		22.386[b]		16.984	
Age	-0.106	-0.186	-0.113	-0.199	-0.032	-0.057
Sex (male)	4.038[a]	0.305	3.756[a]	0.325	4.391[a]	0.380
BMI			0.200	0.201	0.203	0.204
Urinary KB					0.074[a]	0.365
R^2	0.157		0.196		0.307	
F	3.351[a]		2.852		3.759[a]	
ΔR^2	0.157		0.040		0.110	
ΔF	3.351[a]		1.722		5.402[a]	

[a]$P < 0.05$.
[b]$P < 0.01$.
BMI: Body mass index; KB: Ketone bodies.

Differences in KB production might also be due to differences in diet macronutrient composition. A limitation of our study is that we did not record food intake in the first weeks following BMS. However, all patients received standard dietary recommendations, and compliance was reviewed by dieticians at follow-up assessments. Ketosis develops in conditions of reduced glucose availability and marked calorie restriction [29], such as in the first weeks after BMS. Following BMS, protein-rich foods are prioritized over other foods in order to prevent excess loss of fat-free mass[30]. It is

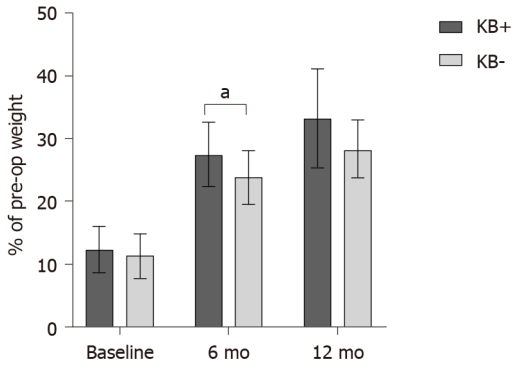

Figure 1 Weight loss at baseline (46.0 ± 13.6 d post-surgery), 6 mo and 12 mo after surgery. KB+: Patients with post-operative ketosis; KB-: Patients without post-operative ketosis. [a]*P* < 0.05.

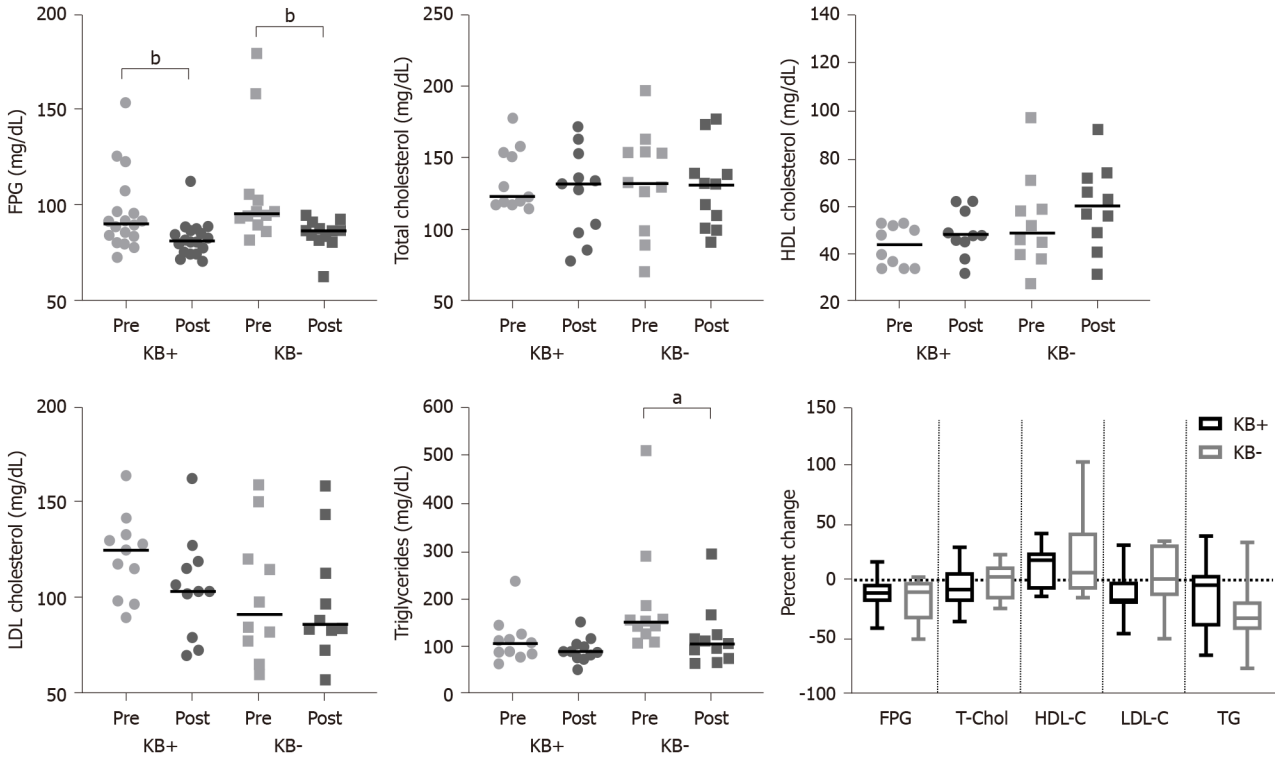

Figure 2 Changes in metabolic parameters at 6 mo after surgery. FPG: Fasting plasma glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; KB+: Patients with post-operative ketosis; KB-: Patients without post-operative ketosis. [a]*P* < 0.05. [b]*P* < 0.01.

unlikely that some patients ingested relatively high amounts of carbohydrates in the first postoperative weeks. On the other hand, it is possible that some greatly restricted carbohydrates to allow adequate protein intake. Deriving energy from proteins is an expensive process for the body, which may lead to calorie consumption and greater WL as compared with diets that rely on carbohydrates as the main energy source[31-33]. In fact, during carbohydrate restriction most of the body's glucose requirements are satisfied by gluconeogenesis from amino acids, a process that requires approximately 400-600 kcal/d[32]. In other settings, several studies have demonstrated that very-low carbohydrate ketogenic diets are associated with greater WL as compared to other dietary regimens[34-36]. Diet composition in the first postoperative weeks might influence subsequent WL even in patients undergoing BMS. Other potential limitations are the relatively small sample size and the availability of data on WL at 12 mo only for a subgroup of patients, which might explain the lack of a statistically significant between-group difference in WL at this timepoint. Finally, we did not formally assess the level of physical activity throughout the 12-month follow-up to detect differences that might influence WL. In general, changes in physical activity during the first 6 mo after BMS (*i.e.*, the timepoint for the assessment of the primary

outcome in this study) are small and unlikely to affect WL[37]. We cannot exclude that changes in physical activity during the following months influenced WL at 12 mo.

CONCLUSION

In conclusion, it is possible that both metabolic status and diet composition influenced KB production in our cohort. Urinary KBs are easy to measure, and could be an early predictor of WL after BMS. Increasing evidence indicates that nutritional ketosis may have several health benefits[2,22,38-49]. Our findings add to this knowledge, suggesting that patients who develop nutritional ketosis following BMS might have greater WL and better metabolic responses to BMS.

ARTICLE HIGHLIGHTS

Research background

Ketone bodies (KB) derived from free fatty acid (FFA) metabolism serve as energy substrates in conditions of reduced glucose availability, but also as metabolic regulators and signalling molecules. Bariatric metabolic surgery (BMS) involves a marked energy deficit that results in massive mobilization of FFAs from adipose tissue, resulting in the activation of ketogenesis. It is not known whether all subjects undergoing BMS become ketotic, and whether there is a relationship between ketogenic capacity and weight loss (WL) following BMS.

Research motivation

We hypothesized that subjects with reduced ketogenic capacity are poorer responders to BMS in terms of WL. Characterization of the relationship between ketogenic capacity and WL following BMS will help understand the metabolic actions of KB and find out whether KB could be used as a predictor of BMS-induced WL.

Research objectives

We assessed the relationship between KB production in the first weeks after BMS and WL at 6 mo. We also assessed the relationship of KB with metabolic parameters and WL at 12 mo.

Research methods

For this retrospective study, we analyzed data from 39 patients who underwent laparoscopic sleeve gastrectomy, had urinary KB measured within two months of surgery and a follow-up of at least 6 mo. KB production was assessed by the presence of acetoacetic acid in urine using an automated dipstick urinalysis. We compared patients who developed post-operative ketosis with those who did not. The relationship of WL at 6 mo with pre-operative anthropometrics, body composition and metabolic parameters, and with post-operative urinary KBs was studied using bivariate correlation analyses. Variables that were significantly correlated were included in a hierarchical multiple-regression analysis, while controlling for sex, age and BMI.

Research results

This was the first study to specifically assess the relationship of ketogenic capacity with weight and metabolic outcomes. Most, but not all patients (69.2%), developed ketosis after a mean of 46.0 ± 13.6 d from surgery. Patients with ketosis were significantly younger [42.9 (37.6; 50.7) years *vs* 51.9 (48.3; 59.9) years, $P = 0.018$] and had significantly lower pre-operative fasting plasma glucose [89.5 (82.5; 96.3) mg/dL *vs* 96.0 (91.0; 105.3) mg/dL, $P = 0.025$] and triglyceride levels [108.0 (84.5; 152.5) mg/dL *vs* 152.0 (124.0; 186.0) mg/dL, $P = 0.020$], but greater LDL cholesterol (121.0 ± 23.5 mg/dL *vs* 100.2 ± 33.9 mg/dL, $P = 0.045$). WL at 6 mo was significantly greater in patients who had developed post-operative ketosis (27.5% ± 5.1% *vs* 23.8% ± 4.3% in the groups with and without ketosis, respectively; $P = 0.035$). At hierarchical multiple regression, urinary KBs and male sex emerged as significant predictors of WL at 6 mo.

Research conclusions

In keeping with the growing body of evidence indicating that nutritional ketosis has

several health benefits, our findings suggest that patients who develop nutritional ketosis following BMS might have greater WL and better metabolic responses to BMS.

Research perspectives

Our findings should be considered hypothesis-generating. Further research is needed to confirm these data in larger populations, and to assess the relationship between ketogenic capacity and metabolic responses to BMS with more sophisticated techniques.

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 January 15; 13(1): 65-69

DOI: 10.4239/wjd.v13.i1.65

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Gut microbiota-derived metabolites are novel targets for improving insulin resistance

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Author contributions: Bastos RMC and Rangel ÉB wrote the letter; Rangel ÉB revised the letter and gave the final approval.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Supported by São Paulo Research Foundation, No. 2013/19560-6 and No. 2017/23195-2; and EFSD (European Foundation for the Study of Diabetes)/Sanofi (to Rangel ÉB).

Country/Territory of origin: Brazil

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

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Abstract

The gut microbiota plays a key role in metabolic diseases. Gut-microbiota-derived metabolites are found in different dietary sources, including: Carbohydrate (acetate, propionate, butyrate, also known as short-chain fatty acids, as well as succinate); protein (hydrogen sulfide, indole, and phenylacetic acid); and lipids (resveratrol-, ferulic acid-, linoleic acid-, catechin- and berry-derived metabolites). Insulin resistance, which is a global pandemic metabolic disease that progresses to type 2 diabetes mellitus, can be directly targeted by these metabolites. Gut-microbiota-derived metabolites have broad effects locally and in distinct organs, in particular skeletal muscle, adipose tissue, and liver. These metabolites can modulate glucose metabolism, including the increase in glucose uptake and lipid oxidation in skeletal muscle, and decrease in lipogenesis and gluconeogenesis associated with lipid oxidation in the liver through activation of phosphatidylinositol 3-kinase - serine/threonine-protein kinase B and AMP-activated protein kinase. In adipose tissue, gut-microbiota-derived metabolites stimulate adipogenesis and thermogenesis, inhibit lipolysis, and attenuate inflammation. Importantly, an increase in energy expenditure and fat oxidation occurs in the whole body. Therefore, the therapeutic potential of current pharmacological and non-pharmacological approaches used to treat diabetes mellitus can be tested to target specific metabolites derived from intestinal bacteria, which may ultimately ameliorate the hyperglycemic burden.

Key Words: Insulin resistance; Gut microbiota; Metabolites; Host metabolism; Metabolic organs; Novel targets

Received: July 3, 2021
Peer-review started: July 3, 2021
First decision: July 28, 2021
Revised: August 1, 2021
Accepted: December 31, 2021
Article in press: December 31, 2021
Published online: January 15, 2022

P-Reviewer: Velikova TV, Yang M
S-Editor: Gao CC
L-Editor: Kerr C
P-Editor: Gao CC

Core Tip: The gut-microbiota-derived metabolites play a key role in metabolic diseases. Insulin signaling pathways are directly targeted by these metabolites, as they promote an increase in glucose uptake and lipid oxidation in skeletal muscle; a decrease in lipogenesis and gluconeogenesis associated with an increase in lipid oxidation in the liver; and an improvement in thermogenesis and inflammation in the adipose tissue. Collectively, these findings pave the way for the development of novel drugs or for investigation of the therapeutic potential of drugs currently used to treat insulin resistance, targeting the gut-microbiota-derived metabolites.

Citation: Bastos RM, Rangel ÉB. Gut microbiota-derived metabolites are novel targets for improving insulin resistance. *World J Diabetes* 2022; 13(1): 65-69
URL: https://www.wjgnet.com/1948-9358/full/v13/i1/65.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i1.65

TO THE EDITOR

We read with interest the recent publication by Jang and Lee[1] on the relationship of mechanisms linking the gut microbiota-derived metabolites to insulin resistance published in this journal.

The gut microbiota plays a key role in metabolic diseases. Gut-microbiota-derived metabolites are found in different dietary sources, including: Carbohydrate (acetate, propionate, butyrate, and succinate); protein (hydrogen sulfide, indole, and phenylacetic acid); and lipids (resveratrol-, ferulic acid-, linoleic acid-, cathecin- and berry-derived metabolites). Insulin signaling pathways are directly targeted by these metabolites. Therefore, gut-microbiota-derived metabolites, in particular, the short-chain fatty acids (SCFAs), increase glucose uptake and lipid oxidation in skeletal muscle, whereas in the liver, SCFAs decrease lipogenesis and gluconeogenesis, increasing the lipid oxidation through activation of phosphatidylinositol 3-kinase - serine/threonine-protein kinase B (PI3K-AKT-PKB) and AMP-activated protein kinase. In adipose tissue, SCFAs stimulate adipogenesis and thermogenesis, inhibit lipolysis, and attenuate inflammation. Therefore, an increase in energy expenditure and fat oxidation occurs in the whole body. Collectively, these findings pave the way for the development of novel drugs or for investigation of the therapeutic potential of drugs currently used to treat insulin resistance, targeting the gut-microbiota-derived metabolites.

Notably, preclinical models and clinical studies substantiate the interaction between intestinal microbiota and the pathophysiology of insulin resistance in type 2 diabetes mellitus (DM)[2-4].

Therefore, this current article provides an overview of the important role of the specific microbiota-derived compounds in insulin-responsive tissues, acting as risk factors or protectors for the development of insulin resistance, and highlights the biologic implications of the muscle–liver–adipose tissue axis interaction.

Even though the authors documented the potential role of some bacterial metabolites as regulators of metabolic functions in the body, such as SCFAs derived from carbohydrates (propionate, butyrate and acetate), and the protein- and lipid-derived metabolites, in modulating pathways of insulin signaling, the impact of these bacterial metabolites on host metabolism warrants further investigation.

Importantly, succinate is a metabolite of the tricarboxylic acid cycle and is produced equally by microbiota and the host[5]. Although this metabolite contributes to improving glucose homeostasis through the activation of intestinal gluconeogenesis [6], in obese individuals, high levels of this circulating metabolite are documented[5]. Furthermore, the imbalance of higher relative abundance of succinate-producing bacteria (Prevotellaceae and Veillonellaceae) and lower relative abundance of succinate-consuming bacteria Odoribacteraceae and Clostridaceae) may promote an increase in succinate levels and, ultimately, impaired glucose metabolism. These authors also pointed out succinate as having a potential role in metabolic-associated cardiovascular disorders and obesity. Additionally, succinate acts as an immunogenic molecule, identified as damage-associated molecular patterns. This molecule is recognized by immune cells and stabilizes hypoxia-inducible factor-1α through its G-protein coupled receptor (succinate receptor 1/SUCNR1 or GPR19), which leads to the

proinflammatory differentiation of T lymphocytes, and production of cytokines through interaction with Toll-like receptor ligands in dendritic cells[7,8]. Collectively, these findings may promote an enhancement of insulin resistance and DM burden.

Furthermore, hydrogen sulfide (H_2S) and the role of sulfur-reducing bacteria from the intestinal microbiota have gained insights into the physiological implications of host glycemic control[9]. Thus, H_2S metabolite may protect against oxidative stress by restoring reduced glutathione (GSH) and scavenging of mitochondrial reactive oxygen species, inducing pro-survival/angiogenesis signaling pathway (STAT3, signal transducer and activator of transcription 3), and promoting immunomodulation (inhibition/activation of nuclear factor-κB) and vasodilation (activation of K_{ATP} ion channel)[10]. However, the balance between therapeutic and harmful effects of H_2S should be considered when targeting that metabolite, as H_2S either endogenous or exogenous, as well as that produced by the gut microbiota, promotes or inhibits a variety of characteristics in mucosal microbiota biofilms[11]. Depending on H_2S concentration, in particular, when the gut microbiota produces an excessive amount, it may cause mucus disruption and inflammation in the colon and contribute to cancer. Conversely, low levels of H_2S directly stabilize mucus layers, prevent fragmentation and adherence of the microbiota biofilm to the epithelium, inhibit the release of invasive opportunistic pathogens or pathobionts, and prevent inflammation and tissue injury[11]. Moreover, H_2S overproduction is a causative factor in the pathogenesis of β-cell death in DM due to increased levels of reactive oxygen and nitrogen species, whereas its deficiency, as a result of increased H_2S consumption by hyperglycemic cells, may lead to endothelial dysfunction, and kidney and heart diseases[12].

As we learn more about gut-microbiota-derived metabolites, we will better understand how to target these metabolites. Thus, acetate, which is involved in host energy, substrate metabolism, and appetite *via* secretion of the gut hormones [glucagon-like peptide (GLP) and peptide YY], may be increased by oral acetate administration (vinegar intake), colonic acetate infusions, acetogenic fibers and acetogenic probiotic administration[13]. These strategies may both decrease whole-body lipolysis and systemic proinflammatory cytokine levels, and increase energy expenditure, insulin sensitivity, and fat oxidation, which contributes to weight control and glucose homeostasis. Probiotics (live microorganisms) act as microbiome modulators and confer a health benefit, as demonstrated by the capacity of selected probiotic strains (lactobacilli and enterococci) to increase SCFA production; in particular, propionate and butyrate[14]. As reviewed elsewhere, probiotic administration (*Bifidobacterium pseudocatenulatum*, *Lactobacillus plantarum*, or the formula VSL#3) in preclinical models of obesity led to an increase in the intestinal barrier function, a reduction in the endotoxemia, acceleration in metabolism, and suppression of body weight gain and insulin resistance *via* modulation of the gut microbiota composition and SCFA production[15]. Probiotics may also ameliorate glucose homeostasis and lipid profile in diabetic mice[15].

From a clinical point of view, obese children treated with the probiotic *Lactobacillus casei* shirota for 6 mo presented with loss of weight, improved lipid metabolism, and an increase in the number of *Bifidobacterium* spp. and acetate concentration in the feces [16]. Likewise, patients with type 2 DM treated with probiotics containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. lactis BB-12 for 6 wk had improved glucose and lipid profiles, which were associated with lower levels of systemic inflammation and increased concentration of acetate[17]. Additionally, modification of gut microbiota by dietary weight loss intervention decreased circulating succinate levels and improved the metabolic profile in a cohort of individuals with type 2 DM and obesity[6].

Pharmacological interventions or xenobiotics may also have effects on gut microbiota. Metformin is the most frequently administered medication to treat patients with insulin resistance and type 2 DM. This drug may alter the gut microbiota composition through an increase in the Bacteroidetes and Verrucomicrobia phyla and the mucin-degrading *Akkermansia muciniphila*, *Bacteroides*, and *Escherichia* genera, as well as in butyrate and propionate production, emphasizing maintenance of the integrity of the intestinal barrier, regulation of bile acid metabolism and improvement in glucose homeostasis[18,19]. Importantly, metformin may have these benefits in newly diagnosed DM[20].

Sodium-glucose cotransporter 2 inhibitors represent the most recently approved class of oral medications for the treatment of type 2 DM. Dapagliflozin decreased the Firmicutes-to-Bacteriodetes ratio in diabetic mice, which was correlated with improvement in vascular function[21]. In a rodent model of type 1 DM, inhibition of SGLT2 reduced the intermediate metabolite succinate and increased butyrate levels, as well as decreased norepinephrine content in the kidney[22]. Hence, the impact of

SGLT2 inhibitors on the gut microbiota is an area of active research.

Likewise, GLP-1 agonists reduced the abundance of the species of the Firmicutes phylum (Lachnospiraceae and Clostridiales) and increased the abundance of the species representing the Proteobacteria (*Burkholderiales bacterium* YL45) and Verrucomicrobia (*Akkermansia muciniphila*), as well as Firmicutes (Clostridiales and Oscillospiraceae) phyla in obese mice[23]. In particular, body weight loss was associated with increased abundance of *Akkermansia muciniphila*, a mucin-degrading SCFA-producing species, whose abundance is decreased in obesity and has a negative correlation with markers of gut permeability and inflammation. Notably, the GLP-1 agonist liraglutide can prevent weight gain by modulating gut microbiota composition in both obese and diabetic obese animals[24].

In the cardiometabolic disease setting, lipid-lowering drugs, such as statins, may also play an important role in modulating gut microbiota. *In vitro* studies have documented increased levels of SCFA production, including propionate, butyrate and acetate[25]. These drugs may increase the abundance of the *Bacteroides*, *Butyricimonas* and *Mucispirillum* genera, which is associated with a decrease in the inflammatory response, including lower levels of interleukin (IL)-1β and IL-6, and higher levels of transforming growth factor β-1 in the ileum, and improved hyperglycemia[26]. In humans, obesity is associated with a microbiota signature based on the abundance of the *Bacteroides* genus profile, displaying the lowest abundances of *Akkermansia* and *Faecalibacterium*, as well as a decrease in the butyrate production potential[27]. Importantly, statin therapy resulted in a lower prevalence of a proinflammatory microbial community type in obese individuals.

In conclusion, the gut microbiota imbalances and maladaptive responses have been implicated in the pathology of insulin resistance, DM, and obesity[28]. Host-gut microbiota interaction is suggested to play a contributory role in the therapeutic effects of antidiabetics, statins, and weight-loss-promoting drugs. Therefore, additional studies combining untargeted metabolomics and proteomics are essential to identify further microbial metabolites or proteins and to determine how they interact with the host targets in improving host metabolism.

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 February 15; 13(2): 70-128

Published by Baishideng Publishing Group Inc

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The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

PUBLICATION DATE
February 15, 2022

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 February 15; 13(2): 70-84

DOI: 10.4239/wjd.v13.i2.70

ISSN 1948-9358 (online)

MINIREVIEWS

Metabolically healthy obesity: Is it really healthy for type 2 diabetes mellitus?

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Author contributions: Wu Q wrote the paper; Xia MF provided the core topic, outlined the manuscript, and modified the paper; Gao X provided the core topic, wrote and revised the manuscript, and provided funding.

Conflict-of-interest statement: Authors declare no conflict of interest for this article.

Supported by Shanghai Municipal Science and Technology Major Project, No. 2017SHZDZX01 (to Gao X).

Country/Territory of origin: China

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

Metabolically healthy obese (MHO) individuals are reported to have a lower risk of developing cardiovascular diseases in comparison with individuals with metabolic syndrome. However, the association between MHO and type 2 diabetes (T2DM) is still controversial. Some studies indicated that MHO is a favorable phenotype for T2DM, but more studies showed that MHO individuals have an increased risk of developing T2DM compared with metabolically healthy normal-weight individuals, especially among those who would acquire metabolically unhealthy obesity. This has been supported by finding insulin resistance and low-grade inflammatory responses in MHO individuals with a tendency for impaired beta-cell dysfunction. Studies also showed that liver fat accumulation increased the risk of incidence of T2DM in MHO. Here, we reviewed current literature on the relationship between MHO and T2DM, discussed the determinants for the development of diabetes in MHO, and summarized the measures for the prevention of T2DM in MHO.

Key Words: Metabolically healthy obesity; Type 2 diabetes; Non-alcoholic fatty liver diseases; Insulin resistance; Low-grade inflammatory status; Beta-cell dysfunction

Core Tip: Metabolically healthy obese individuals have already developed impaired insulin sensitivity with dysfunction of insulin action on subcutaneous tissue, as well as a tendency for beta-cell dysfunction and a chronic low-grade inflammatory status compared with metabolically healthy normal-weight individuals. Thus, it is an

Received: February 26, 2021
Peer-review started: February 26, 2021
First decision: April 20, 2021
Revised: April 27, 2021
Accepted: January 20, 2022
Article in press: January 20, 2022
Published online: February 15, 2022

P-Reviewer: Belete R
S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

unfavorable phenotype for type 2 diabetes, with metabolic changes preceding the incidence of diabetes. Liver fat content might be an important contributor to the development of diabetes in metabolically healthy obesity among all risk factors. More attention should be paid to the weight management and metabolic status of these individuals.

Citation: Wu Q, Xia MF, Gao X. Metabolically healthy obesity: Is it really healthy for type 2 diabetes mellitus? *World J Diabetes* 2022; 13(2): 70-84
URL: https://www.wjgnet.com/1948-9358/full/v13/i2/70.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i2.70

INTRODUCTION

Obesity and diabetes have been growing public health problems for decades. The prevalence of obesity had doubled worldwide in 2015 compared with that in 1980[1]. Individuals with obesity are generally likely to develop type 2 diabetes mellitus (T2DM), since obesity is linked to increased risk of insulin resistance, beta-cell dysfunction, and imbalanced fat tissue metabolism[2]. However, there is a subset of obese individuals who are at low risk of cardiovascular disease with a relatively normal metabolic profile compared with metabolic unhealthy obesity (MUO) individuals, a condition known as metabolically healthy obesity (MHO)[3]. Some studies showed that MHO individuals were not at increased risk for diabetes compared with those who are classified as metabolically healthy normal weight (MHNW)[4,5], but others indicated that MHO was associated with an increased risk of developing T2DM over a lifetime than MHNW[6,7]. Whether MHO is a real health status, or more specifically, whether it predisposes individuals to T2DM, is still controversial.

In this review, we address the above questions by discussing controversies related to metabolically healthy obesity, including the causal relationship between MHO and T2DM and its related diseases as well as the underlying mechanisms.

PREVALENCE OF METABOLICALLY HEALTHY OBESITY

MHO was described by Sims in 2001 as obesity with the absence of metabolic syndrome and metabolic complications[8]. Most definitions of MHO are based on the criteria for metabolic syndrome based on the definition provided by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP-III)[9], which include: (1) The presence of central obesity, waist circumference ≥ 102 cm (90 cm for Asians) in men and ≥ 88 cm (80 cm for Asians) in women; (2) Systolic blood pressure ≥ 17.3 kPa (130 mmHg) and/or diastolic blood pressure ≥ 11.3 kPa (85 mmHg); (3) Triglycerides ≥ 1.7 mmol/L (150 mg/dL); (4) Fasting blood glucose ≥ 5.6 mmol/L (100 mg/dL); and (5) High-density lipoprotein cholesterol (HDL-C) less than 1.03 mmol/L (40 mg/dL) in men or less than 1.30 mmol/L (50 mg/dL) in women. Most definitions of MHO require fewer than two or the absence of any metabolic abnormalities except for waist circumference[7,10-13]. However, the details of the MHO definitions are slightly different. One study defined MHO as individuals who possess no more than two of four metabolic abnormalities except waist circumference[14]. Some researchers believe that those who use anti-hypertension drugs, lipid-lowering agents, or glucose-lowering medicines are also metabolically abnormal even though their metabolic levels are good[15,16]. The level of low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) were also included in the definition of MHO by Karelis *et al*[17]. Insulin resistance evaluated by the homeostasis model assessment for insulin resistance (HOMA-IR) and inflammatory status expressed by C-reactive protein (CRP) has been added to the criteria for MHO by Wildman *et al*[18]. Lwow *et al*[19] proposed using a combined lipid accumulation product with the criteria mentioned above as new criteria for MHO. Smith *et al*[20] decreased the cut point of triglyceride to a level of 95 mg/dL and includes the criteria for the evaluation of intrahepatic lipid content. MHO was also defined as the absence of metabolic diseases such as hypertension, T2DM, and dyslipidemia[15]. The detailed information of common definitions of

MHO was showed in Table 1.

The prevalence of MHO differs from 2.2% to 11.9% in the general population according to the different definitions of MHO[21]. The prevalence of MHO in Americans from the National Health and Nutrition Examination Survey was 19.9% when metabolic health was defined as the absence of components of NCEP ATP-III; the prevalence decreased to 16.0% when the threshold of glucose was reduced to 100 mg/dL, and it decreased to 14.8% when HbA1c was included in the definition of MHO. The prevalence further decreased to 12.2% when the cut-off point of blood pressure was reduced from 17.3/11.3 kPa (130/85 mmHg) to 16.0/10.6 kPa (120/80 mmHg)[22]. Using the criteria of less than three components of NCEP ATP-III, the prevalence of MHO was 8.6% in Spanish[23] and 10.3% in China[24]. There was an age-related reduction in the proportion of MHO regardless of different definitions[24]. Besides, obese patients with higher body mass index (BMI) levels had a lower proportion of MHO, which accounted for 53.7% of participants with BMI at 30-34.9 kg/m^2 and 4.9% of participants with BMI at 35-39.9 kg/m^2[25]. When a more stringent criterion of having no components of NECP ATP-III was applied to the definition of MHO, there was no metabolic healthy individual with BMI ≥ 35 kg/m^2[23]. It means that there might be a cut-off point in individuals with MHO, beyond which their metabolic status would no longer be healthy.

Metabolically healthy individuals will develop metabolic disorders over time. Feng *et al*[7] discovered that only 42.84% of individuals in a group of MHO remained metabolic healthy after a 4-year follow-up. Gilardini *et al*[26] reported that 44% of MHO became metabolically unhealthy after 6-year follow-up, and the proportion increased to 62% after 12-year follow-up. The proportion of transition from MHO to MUO might differ because of different definitions of MHO and various lengths of follow-up[27]. Generally speaking, MHO is not a health status according to the current definitions of having one or two abnormal conditions but rather a transient state that can transition to an unhealthy state over time. Thus, it is fundamentally inaccurate to define those groups of people as "healthy" and worthwhile to investigate the relationship between MHO and T2DM.

RISK OF T2DM IN MHO SUBJECTS

The association between T2DM and MHO has been studied with diverse results, as shown in Table 2. Although MHO is believed to be a healthier phenotype for T2DM when compared with metabolically unhealthy normal weight and MUO individuals, most of the current studies supported that MHO phenotype relates to an increased incidence of T2DM in cohort studies compared to MHNW individuals, independent of the length of follow-up[7,16,28-31]. Wei *et al*[30] examined 17801 individuals in the Dongfeng-Tongji cohort study and showed that the hazard ratio [95% confidence interval (CI)] of diabetes for MHO was 1.74 (1.16-2.59). The multivariate-adjusted hazard ratio (95%CI) of diabetes for MHO without non-alcoholic fatty liver diseases (NAFLD) was 1.57 (1.14-2.16) after an average 4.1-year follow-up in The Kangbuk Samsung Health Study[16]. However, studies have also found that different sub-groups of MHO individuals have different risks of developing diabetes at follow-up [14,30,32,33]. For example, Wang *et al*[33] found that an MHO phenotype that is stable over time is not significantly related to an increased risk of incident diabetes in a 6-year follow-up cohort study when compared with the MHNW phenotype, while the majority of MHO participants had an increased risk of developing diabetes over their lifetimes. Consistently, our human data from Shanghai Changfeng Study showed a similar result that MHO individuals who transition into MUO had a higher risk of developing T2DM while there was no significant association between MHO and incidence of diabetes in the whole population (unpublished data). Thus, it will be of great importance to investigate the determinants related to incident diabetes in MHO individuals.

Several factors might contribute to the development of T2DM in the MHO participants. Baseline body weight is an important factor associated with the high risk of incidence of diabetes. It is universally known that obesity can increase the risk of T2DM. One study found that obese individuals (BMI ≥ 30 kg/m^2) with a healthy metabolic status were at greater risk of developing diabetes than either overweight or normal-weight subjects, and the risk was in proportion to the degree of obesity[14,34]. The previous study has also shown that all metabolically unhealthy individuals, regardless of their body weight, have a higher risk of diabetes[14]. Unstable MHO individuals who progress into unhealthy metabolic statuses also have an elevated risk

Table 1 Definitions of metabolic health in previous publications

Ref.	BP, kPa (mmHg)	Plasma glucose, mmol/L	TG, mmol/L	HDL-C, mmol/L	LDL-C, mmol/L	TC, mmol/L	WC, cm	Insulin sensitivity	CRP, mg/L	Intrahepatic lipid content	Others	Metabolic health
NECP ATP III [9]	SBP ≥ 17.3 (130) and/or DBP ≥ 11.3 (85) and/or treatment	FPG ≥ 5.60	≥ 1.70	< 1.29 in women, < 1.03 in men	-	-	> 88 in women, >102 in men	-	-	-	-	< 3 of above
Karelis *et al*[17]	-	-	≤ 1.70	≥ 1.30 and no treatment	≤ 2.60 and no treatment	≤ 5.20	-	HOMA-IR ≤ 1.95	-	-	-	> 3 of above
Meigs *et al*[4]	SBP ≥ 17.3 (130) or DBP ≥ 11.3(85) or treatment	5.6 < FPG ≤ 6.9	≥ 1.70	< 1.30 in women, < 1.00 in men	-	-	> 88 in women, > 102 in men	-	-	-	-	< 2 of above
Meigs *et al*[4]	-	-	-	-	-	-	-	HOMA-IR ≥ 75th percentile	-	-	-	None of above
Aguilar-Salinas *et al*[89]	SBP > 18.6 (140) and/or DBP > 12.0 (90) and/or treatment	FPG ≥ 7.0, or 2-h OGTT ≥ 11.1, or RBG ≥ 11.11 or treatment	-	< 1.04	-	-	-	-	-	-	-	None of above
Wildman *et al* [18]	SBP ≥ 17.3 (130) or DBP ≥ 11.3 (85) or treatment	FPG ≥ 5.56 or treatment	≥ 1.70	< 1.30 in women, < 1.04 in men or treatment	-	-	-	HOMA-IR > 90th percentile	> 90th percentile	-	-	< 2 of above
van Vliet-Ostaptchouk *et al*[90]	SBP ≥ 17.3 (130) or DBP ≥ 11.3 (85) or treatment	FPG ≥ 6.10 or treatment or history/diagnosis of type 2 diabetes	≥ 1.70 or treatment	< 1.03 in men or < 1.30 in women or treatment	-	-	-	-	-	-	-	<2 of above
Jana V van Vliet-Ostaptchouk *et al*[90]	SBP ≥ 18.6 (140) or DBP ≥ 12.0(90) or treatment	FPG ≥ 7.0 or treatment or history/diagnosis of type 2 diabetes	≥ 1.70 or treatment	< 1.03 in men or < 1.30 in women or treatment	-	-	-	-	-	-	-	< 2 of above
Smith *et al*[20]	SBP < 17.3 (130) and/or DBP < 11.3 (85)	FPG < 5.60, or 2-h OGTT glucose < 7.80	< 1.07	≥ 1.29 in women, ≥ 1.04 in men	-	-	-	GIR > 8 mg/kg FFM/min during an HECP (insulin infusion rate: 40 mU/m^2/min)	-	< 5% of liver volume by imaging or < 5% of hepatocytes with intracellular TG by histology	Basic criteria: Absence of diagnosis or therapy of cardiometabolic diseases	all of above

BP: Blood pressure; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; OGTT: Oral glucose tolerance test; RBG: Random blood glucose; HbA1c: Glycosylated hemoglobin A1c; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol; WC: Waist circumference; HOMA-IR: Homeostasis model assessment of insulin resistance; GIR: Glucose infusion rate; HECP: Hyperinsulinemic-euglycemic clamp procedure; hs-CRP: High-sensitivity C-reactive protein.

Table 2 Cohort studies of the association of metabolically healthy obesity and type 2 diabetes in the last 5 years

Ref.	Definition of "metabolic health"	MHO, *n*	Main findings
Wei *et al*[30], 2020	Having < 2 of the following criteria: (1) TG ≥ 1.7 mmol/L or lipid-lowering drugs; (2) SBP ≥ 17.3 kPa (130 mmHg) or DBP ≥ 11.3 kPa (85 mmHg) or anti-hypertensive drugs; (3) FPG ≥ 5.6 mmol/L; and (4) HDL-C < 1.04 mmol/L for men and < 1.29 mmol/L for women.	693	MHO was associated with an increased incidence of diabetes, and the association did not differ by the presence or absence of NAFLD.
Feng *et al*[7], 2020	Having < 2 of the following criteria: (1) Hyperglycemia, defined as FPG ≥ 5.6 mmol/L (100 mg/dL); (2) Elevated blood pressure, defined as SBP ≥ 17.3 kPa (130 mmHg) and/or DBP ≥11.3 kPa (85 mmHg) or antihypertensive drug treatment; (3) Hypertriglyceridemia, defined as TG ≥ 1.7 mmol/L (150 mg/dL); and (4) Reduced HDL-C levels, defined as drug treatment to increase HDL-C levels.	3728	The MHO phenotype was associated with an increased incidence of diabetes in older adults. The presence of metabolic disorders in the group with MHO was associated with increased diabetes risk and was predicted by the waist circumference at baseline.
Kim *et al*[32], 2019	Having two or fewer metabolic abnormalities as follows: (1) WC ≥ 90 cm in men and ≥ 85 cm in women; (2) SBP ≥ 17.3 kPa (130 mmHg) or DBP ≥ 11.3 kPa (85 mmHg) or medication use; (3) FPG ≥ 5.6 mmol/L (100 mg/dL) or claim for T2DM or on anti-diabetic medications; (4) Hypertriglyceridemia ≥ 1.7 mmol/L (150 mg/dL) or on lipid medications; and (5) HDL-C < 1.04 mmol/L (40 mg/dL) in men and < 1.29 mmol/L (50 mg/dL) in women, or medication use.	796371	MHO and MHNW phenotypes were transient phenotypes, and their change into metabolic unhealthy status was an important risk factor for the development of T2DM both in obese and normal-weight subjects. Transition into a metabolically unhealthy phenotype was a more significant risk factor of developing T2DM than obesity itself.
Wang *et al*[33], 2018	Having < 2 of the following criteria: (1) SBP ≥ 17.3 kPa (130 mmHg) or DBP ≥ 11.3 kPa (85 mmHg) or current treatment for hypertension; (2) Fasting TG level ≥ 1.7 mmol/L; (3) HDL-C level < 1.03 mmol/l for males or < 1.29 mmol/L for females; and (4) FPG ≥ 5.60 mmol/L.	2153	Stable metabolically healthy overweight/obesity Individuals and those who transitioned to the metabolically healthy status from MUNW did not have an increased risk of incident T2DM. Participants who transitioned from the metabolically healthy overweight/obesity to metabolically unhealthy overweight/obesity phenotype and stable MUNW phenotype showed an increased risk of incident T2DM.
Fingeret *et al* [31], 2018	Having two or fewer metabolic abnormalities as follows: (1) FPG ≥ 5.6 mmol/L or drug treatment; (2) Fasting TG ≥ 1.7 mmol/L or drug treatment; (3) Fasting HDL-C < 1.30 mmol/L in women and < 1.00 mmol/L in men or drug treatment; (4) SBP ≥ 17.3 kPa (130 mmHg), DBP ≥ 11.3 kPa (85 mmHg), or drug treatment; and (5) WC ≥ 102 cm for men and ≥ 88 cm for women.	170	MHO leads to a higher risk of developing cardiovascular risk factors such as hypertension, diabetes, dyslipidemia as compared with MHNW. MHO is transient and should be regarded by clinicians as a warning sign.
Liu *et al*[91], 2018	Having < 2 of metabolic abnormalities as follows: (1) TG ≥ 1.7 mmol/L; (2) HDL-C < 1.0 mmol/L; (3) SBP ≥ 17.3 kPa (130 mmHg) and/or DBP ≥ 11.3 kPa (85 mmHg); and (4) FPG ≥ 5.6 mmol/ L (≥ 100 mg/dL).	1184	MHO and MUNW phenotypes had an increased risk for diabetes. Both baseline metabolic status and follow-up changes played more important roles than obesity for diabetes incidence after adjusted for potential confounding factors. MHO is a transient condition.
Janghorbani *et al*[29], 2017	Having none of metabolic abnormalities as follows: (1) TG ≥ 1.7 mmol/L (150 mg/dL); (2) HDL < 1.04 mmol/L(40 mg/dL) in men and < 1.29 mmol/L(50 mg/dL) in women; (3)BP ≥ 17.3/11.3 kPa (130/85 mmHg) or on antihypertensive medication; and (4) FPG ≥ 5.6 mmol/L (100 mg/dL).	75	Metabolic abnormalities increased risk for incident T2D at any BMI status. Also, obesity is a risk factor for the incidence of T2DM, even in the absence of any metabolic abnormalities.
Latifi *et al*[25], 2017	Having none of metabolic abnormalities as follows: (1) WC ≥ 102 cm in men and ≥ 88 cm in women; (2) TG ≥ 1.7 mmol/L (150 mg/dL) or drug use; (3) HDL < 1.04 mmol/L (40 mg/dL) in men and 1.29 mmol/L (50 mg/dL) in women or drug consumption for hyperlipidemia; (4) BP ≥ 17.3/10.6 kPa (130/80 mmHg) or a history of anti-hypertensive drug consumption; and (5) FPG ≥ 5.6 mmol/L (100 mg/dL), or a history of diabetes mellitus or consumption of anti-diabetes drugs.	NA	There was a specific higher risk of developing metabolic syndrome and diabetes in MHO.
Navarro-Gonzalez *et al* [14], 2016	Having < 3 of the following criteria: (1) TG ≥ 1.7 mmol/L (150 mg/dL); (2) HDL-C > 1.04 mmol/L (40 mg/dL) for men and > 1.29 mmol/L (50 mg/dL) for women; (3) BP ≥ 17.3/11.3 kPa (130/85 mmHg); or (4) FPG ≥ 5.6 mmol/L (100 mg/dL). All individuals currently taking a pharmacological treatment for hypertension were assumed to have raised BP.	389	MHO individuals had an increased risk of incident type 2 diabetes but mainly among those who progressed MUO. MHO individuals who remained with one or no metabolic health risk factors or lost weight overtime did not have a significant risk of diabetes. Metabolically unhealthy individuals had a greater risk of diabetes compared with subjects with MHO.
Guo *et al*[3], 2016	Having all three components as follows: (1) Untreated SBP < 17.3 kPa (130 mmHg) and DBP < 11.3 kPa (85 mmHg); (2) Untreated FPG < 5.6 mmol/L (100 mg/dl) or HbA1c < 5.7%; and (3) Untreated TC < 6.2 mmol/L (240 mg/dL) and HDL ≥ 1.04 mmol/L (40 mg/dL) in men and ≥ 1.29 mmol/L (50 mg/dL) in women.	260	People with healthy obesity have lower risks for diabetes, coronary heart disease, stroke, and mortality compared with unhealthy subjects regardless of their BMI status. Obesity did not affect the risks of coronary heart disease, stroke, and mortality, but did increase diabetes risk.

Jung *et al*[40], 2016	Having < 2 of the following criteria: (1) SBP ≥ 17.3 kPa (130 mmHg) and/or a DBP ≥ 11.3 kPa (85 mmHg), or on antihypertensive treatment; (2) TG ≥ 1.7 mmol/L; (3) FPG ≥ 5.6 mmol/L (impaired fasting glucose, IFG); (4) HDL-C < 1.0 mmol/L in men and < 1.3 mmol/L in women; (5) HOMA-IR ≥ 90th percentile (≥ 2.91); and (6) Hs-CRP ≥ 90th percentile (≥ 2.0 mg/L).	4635	MHO subjects have a substantially increased risk of incident type 2 diabetes compared with MHNO subjects in an Asian population. The presence of FLD assessed by FLI partially explains this increased risk.
Chang *et al*[16], 2016	Having none of the following criteria: (1) BP ≥ 17.3/11.3 kPa (130/85 mmHg) or current use of blood pressure-lowering agents; (2) FPG ≥ 5.6 mmol/L (100 mg/dL) or current use of blood glucose-lowering agents; (3) TG ≥ 1.7 mmol/L (150 mg/dL) or current use of lipid-lowering agents (15); (4) HDL-C < 1.04 mmol/L (40 mg/dL) in men or < 1.29 mmol/L (50 mg/dL) in women; or (5) Insulin resistance, defined as HOMA-IR score ≥ 2.5.	8140	Metabolically healthy overweight and obese individuals were both associated with an increased incidence of diabetes, even in the absence of NAFLD. Obese phenotype itself can drive the development of diabetes, even in the absence of metabolic abnormalities and NAFLD.
Ryoo *et al*[34], 2015	Having < 2 of the following criteria: (1) SBP ≥ 17.3 kPa (130 mmHg) and/or DBP ≥ 11.3 kPa (85 mmHg); (2) TG ≥ 1.7 mmol/L; (3) FPG ≥ 5.6 mmol/L; (4) HDL-C < 1.0 mmol/L; and (5) HOMA-IR ≥ 90th percentile.	240	The risk for diabetes was in proportion to both metabolic health status and degree of obesity in Korean men. Additionally, metabolically healthy status was a more significant determinant for the development of diabetes than obesity itself.

TG: Triglycerides; BP: Blood pressure; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; HOMA-IR: Homeostatic model assessment of insulin resistance; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; hs-CRP: High sensitivity C reactive protein; WC: Waist circumference; T2DM: Type 2 diabetes mellitus; MHO: Metabolically healthy obesity; MUO: Metabolically unhealthy obesity; MHNW: Metabolically healthy normal weight; MUNW: Metabolically unhealthy normal weight; NAFLD: Non-alcoholic fatty liver disease; FLI: Fatty liver index.

of developing diabetes. Weight gain was a risk factor for the progression from a healthy condition to an unhealthy one, which further develops into T2DM. In one study, MHO individuals who developed cardiometabolic risk complications gained 6% ± 14% of their body weight (4.9 ± 11.8 kg) compared to 5% ± 14% (3.9 ± 11.3 kg) for those that retained a healthy status[35]. Besides, MHO participants with larger waist circumference at baseline are more likely to transition into an unhealthy phenotype[7]. This has been supported by studies showing that visceral abdominal fat accumulation and fatty liver in MHO contribute to this transition[12,36,37]. Thus, MHO individuals with high liver fat content or large waist circumference are possibly associated with a high risk of diabetes as they have a trend to transferring into MUO phenotype. Our previous study found that visceral adipose area measured by visceral adiposity index in Chinese adults has a more favorable function to predict the development of diabetes than BMI and waist circumference in MHO individuals[38]. Some researchers found that MHO individuals with a high fatty liver index[39] have an increased risk of incident T2DM[40].

LIVER FAT ACCUMULATION IS CRUCIAL FOR DETERMINING THE DEVELOPMENT OF T2DM IN MHO

NAFLD is believed to be significantly associated with the long-term risk of T2DM, and increased liver fat can predict the incidence of T2DM independent of obesity[41,42]. Bian *et al*[43] found that elevated liver fat content (LFC) showed a positive association with insulin resistance and a higher level of nocturnal mean blood concentration before the onset of diabetes. The presence of NAFLD will promote the transition from MHO to a metabolic unhealthy state, and further increases the long-term risk of

incidence of T2DM and even aggravates the deterioration of liver diseases in MHO. Hwang *et al*[12] found that the presence of NAFLD in MHO could predict the conversion from a metabolic health status into a metabolic unhealthy status independent of age, sex, BMI, lifestyle factors, components of metabolic syndrome, and insulin resistance evaluated by HOMA-IR. This result was supported by Hashimoto *et al*[37] with findings that fatty liver index was a predictor for the transition from MHO to MUO phenotype even adjusted for body weight change. However, Hwang *et al*[12] also found that the association between the NAFLD and future transition of MHO into MUO weakened as BMI increased, and the relationship was more prominent in lower BMI individuals. Studies also found that the risk of NAFLD, non-alcoholic steatohepatitis, and liver fibrosis increased as BMI elevated in MHO[16,44]. The unstable MHO status predicted by NAFLD would increase the risk for the development of T2DM, as mentioned above, and therefore the presence of NAFLD in MHO might increase the risk of incident T2DM. Chang *et al*[16] supported this with the result that the risk of incidence of T2DM in MHO subjects with NAFLD increased compared to those free of NAFLD. Ampuero *et al*[45] also found that MHO individuals with biopsy-proven NAFLD or with an intermediate-to-high risk of significant fibrosis evaluated by Hepanet Fibrosis Score (> 0.12) were at risk of developing T2DM.

However, despite the presence of elevated LFC in MHO increasing the risk for the transition of MHO and the incidence of T2DM, few studies regarded intrahepatic lipids content as one of the criteria for the definition of metabolic health. Our previous study found that LFC was positively associated with metabolic disorders independent of related anthropometric and metabolic parameters, and the risk for metabolic diseases increased in an LFC-dependent manner when LFC ≥ 5%[46]. Besides, part of normal individuals without metabolic disorders had a higher LFC[46]. Hence, we agree with Smith *et al*[20] that the evaluation of LFC should be regarded as another crucial criterion for defining "metabolic health".

ASSOCIATION BETWEEN MHO AND METABOLIC DISEASES RELATED TO T2DM

Cardiovascular disease

Studies have found that subjects with MHO have a lower risk of cardiovascular disease (CVD) than MUO individuals over their lifetimes but still have a higher risk than MHNW subjects[47-50]. The transition to an unhealthier metabolic status and the longer duration of unhealthy metabolic conditions contribute to the increased risk of developing CVD among MHO subjects[50-52]. Furthermore, the risk of developing CVD for MHO subjects who initially develop diabetes, hypertension, or hypercholesterolemia tends to be higher than in MHNW subjects[53]. Obesity might increase the risk of CVD independently. A meta-analysis concluded that CVD risk is increased in metabolically healthy overweight or obese participants than in MHNW individuals even when there are no metabolic risk factors[54]. Similarly, obese individuals have been reported to be at higher risk of coronary heart disease irrespective of metabolic health, which challenges the concept of "metabolically healthy obesity"[55].

Chronic kidney disease

Previous studies have shown an increased risk of developing chronic kidney disease (CKD), defined as an estimated glomerular filtration rate of less than 60 mL/min/1.73 m^2 in metabolically healthy overweight/obese subjects compared to MHNW individuals at follow-up, with metabolic health judged as having less than two metabolic abnormalities[56]. Another study showed a similar result in which MHO individuals with no metabolic abnormalities had a higher risk of developing CKD, and this risk was greater in those 40 years or older than in the young[57]. Systemic inflammation measured by high sensitivity-CRP (hs-CRP) might partially contribute to the association between MHO and CKD[11]. Furthermore, individuals who progress to MUO at follow-up show a higher risk of CKD compared with remaining MHO subjects[58,59]. However, Chen *et al*[60] found that the risk difference was not significant in MHO subjects compared to MHNW individuals in the early stage of CKD. This discrepancy might come from the different definitions of CKD, as Chen *et al*[60] combined proteinuria and structural changes in the kidney as indicators.

POSSIBLE MECHANISMS OF THE FUTURE INCIDENCE OF T2DM IN MHO

The possible mechanisms underlying the pathophysiology of incident T2DM in MHO include beta-cell dysfunction, insulin resistance, leptin and adiponectin imbalance, as well as a chronic low-grade inflammatory status (Figure 1). The presence of NAFLD in MHO is also an important factor for the development of T2DM.

Impaired insulin action and insulin resistance

Mature insulin and C-peptide are produced from the precursor proinsulin, and increased proinsulin is observed in insulin-resistant and/or glucose-intolerant individuals[61]. Significantly increased levels of plasma proinsulin, split proinsulin, and C-peptide are observed in MHO subjects compared to MHNW subjects[62]. A similar result has been found in a Chinese population, in which the serum insulin of MHO subjects is significantly elevated[63]. Studies have confirmed the above results showing that HOMA-IR evaluations are significantly different between MHO and MHNW subjects, with a higher value of HOMA-IR in MHO individuals[62,63].

The action of insulin on subcutaneous adipocytes is impaired as well. Rydén *et al*[10] compared the inhibitory action on lipolysis and the stimulatory effect on lipogenesis of insulin in metabolically healthy subjects who were lean, overweight, or obese and found that insulin resistance was already observed in metabolically healthy overweight and obese subjects. In the classical agonist-receptor interaction model, the half-maximum effects for insulin to inhibit adipocyte lipolysis and lipogenesis in overweight/obese people were 10 times and 100 times higher than that in lean people, respectively. The above model suggested that alterations in intracellular events downstream of the insulin receptor and their initial signaling steps have already happened in those individuals. The decreased expression of the insulin signaling mediator *AKT2* might partially explain the increased maximum concentration of insulin hormones needed for an antilipolytic effect and lipogenesis, as AKT2 is an early signaling factor common to the two pathways[64]. Furthermore, the impaired lipogenic function might in part result from a decrease in *SLC2A4* (glucose transporter type 4) mRNA expression, which is essential for insulin-induced glucose uptake by fat cells and stimulating lipogenesis[64]. When testing the maximum insulin action on subcutaneous adipocytes, Rydén *et al*[10] found that the lipogenic effect of insulin hormone was reduced by more than 50% in healthy overweight/obese subjects comparing to lean individuals, and the effect was further impaired in the unhealthy obese groups. Thus, there are reasons to believe that insulin resistance is already present in MHO individuals.

Beta-cell dysfunction

There is no apparent evidence that beta cells in MHO subjects are severely impaired, but they may be partially impaired according to previous studies. Hjelmgren *et al*[62] found that MHO individuals are at increased risk for having β-cell dysfunction, as evaluated by proinsulin levels > 11 pmol/L compared to MHNW subjects, with a relative risk of 18.2 (95%CI: 2.1-159.3). However, Zhao *et al*[63] failed to find a significant difference in HOMA-β between MHNW and obese subjects, though the value of HOMA-β in MHO tended to be higher than that in MHNW individuals among middle-aged subjects. The discrepancy between the two studies might come from their different definitions of metabolic health, differences in race and age, and the relatively small sample size in Zhao's study for evaluating statistical differences. Overall, studies on beta-cell dysfunction are too few to confirm their impaired function in MHO individuals.

Immune and inflammatory responses

Obesity has always been believed to be a chronic low-grade inflammatory status[65], referred to as meta-inflammation. This chronic low-grade inflammation is believed to be a central link between obesity and T2DM[66,67]. A previous study showed that meta-inflammation is presented in MHO subjects as well[68].

Macrophage infiltration in adipose tissue causes increased proinflammatory cytokines and contributes to the development of insulin resistance and T2DM[69]. Christou *et al*[70] found that circulating inflammatory intermediate monocytes [Mon2 ($CD14^{++}CD16^{+}$)] are upregulated in MHO individuals, and nonclassical monocytes [Mon3 ($CD14^{+}CD16^{++}$)] tended to be higher in comparison to metabolically healthy lean individuals when metabolic health was defined as fewer than two metabolic disabilities. The absolute counts of nonclassical Mon3 showed a positive association with HOMA-IR in that study. However, that result differed from previous studies, as

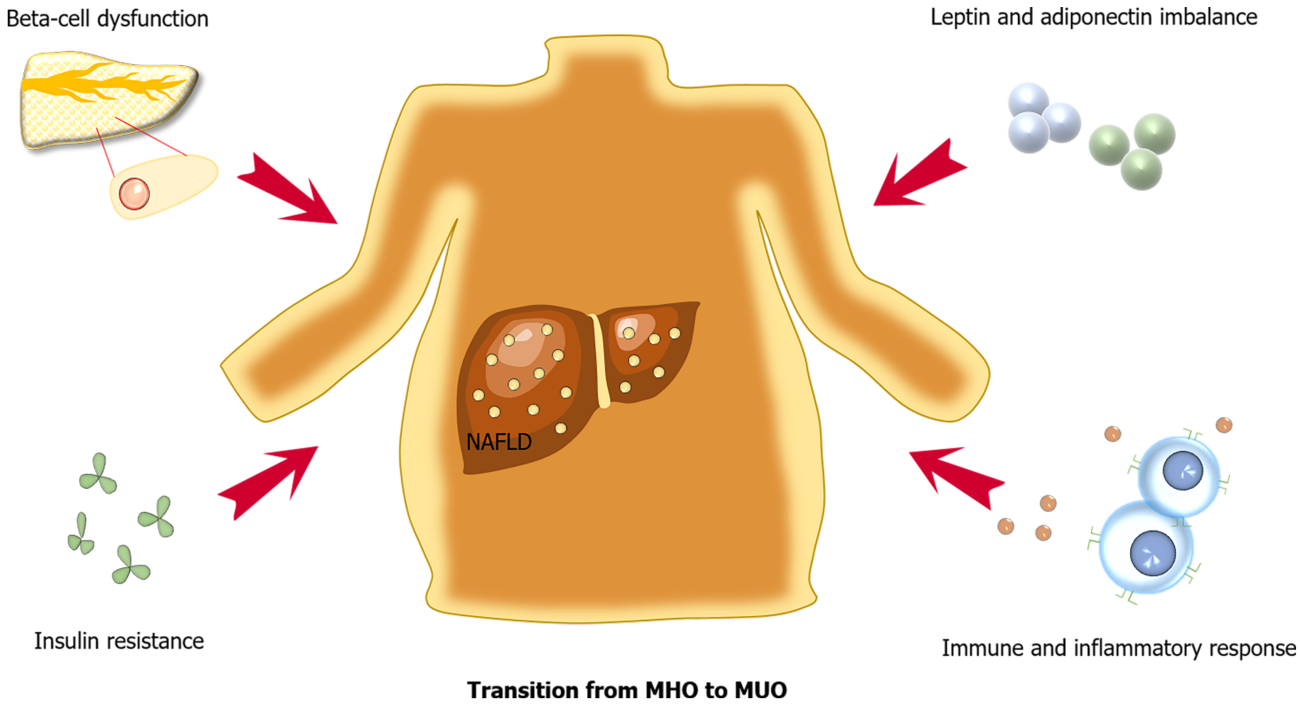

Figure 1 Possible mechanisms that contribute to the future incidence of type 2 diabetes mellitus in the transition from metabolically healthy obesity to metabolically unhealthy obesity. The presence of non-alcoholic fatty liver disease in metabolically healthy obesity is crucial to the incidence of type 2 diabetes mellitus. The possible mechanisms underlying the future development of type 2 diabetes mellitus in metabolically healthy obesity include beta-cell dysfunction, insulin resistance with impaired insulin action, adiponectin concentration reduction, as well as a chronic low-grade inflammatory status. MHO: Metabolically healthy obesity; MUO: Metabolically unhealthy obesity; NAFLD: Non-alcoholic fatty liver disease.

the participants they recruited were taking antidiabetic medications, which might have disturbed the relationship between Mon3 and the level of insulin resistance[71,72].

A previous study found that an imbalance of T cell subsets is responsible for the pathogenesis of obesity and T2DM[68]. Th22 subsets might play a role in obesity and T2DM progression, with MHO and T2DM individuals having significantly elevated peripheral blood Th22 frequencies[73]. This might partially result from the significantly increased transcription of aryl hydrocarbon receptor (AHR), a transcription factor responsible for the differentiation of Th22, on peripheral blood mononuclear cells in both obese and T2DM individuals compared with metabolically healthy normal BMI subjects. AHR is significantly positively associated with elevated hs-CRP and HOMA-IR levels in MHO individuals. Although it was tested in peripheral blood mononuclear cells and not T cells in that study, AHR expression in peripheral blood mononuclear cells is more likely to be a causative factor in Th polarization with a currently unknown mechanism[63].

Leptin and adiponectin

Adipose tissue is not only an energy storage depot, it also has endocrine functions and produces some cytokines that influence metabolism throughout the human body. White fat tissue can participate in regulating insulin sensitivity, lipid metabolism, and low-grade inflammation[74,75]. Leptin and adiponectin are important factors in these conditions. Leptin is responsible for food intake and metabolism regulation, while adiponectin release contributes to energy metabolism, insulin action, lipid metabolism regulation, and oxidative stress. Increased adiponectin is associated with better insulin sensitivity in the human body[76]. A previous study found that adiponectin is significantly decreased in MHO Han Chinese adolescents compared with a normal-weight control group, and a similar result was also found in middle-aged Norwegians [77,78]. Thus, insulin sensitivity might be disturbed in MHO individuals with elevated adiponectin. However, Carvalho *et al*[79] found an inconsistent result that the serum adiponectin concentration in MHO subjects had no significant difference with MHNW individuals. The small sample size of the latter study might have contributed to the inability to find statistically significant differences in adiponectin. Taken together, most studies indicated an increased leptin/adiponectin ratio in MHO compared to MHNW individuals[77-79], which was already regarded as a sensitive indicator of metabolic syndrome and insulin sensitivity[80]. Thus, no matter whether adiponectin

is decreased in MHO individuals, it can be deduced that insulin sensitivity has been already impaired in MHO subjects with an elevated leptin/adiponectin ratio.

WEIGHT CONTROL MIGHT IMPROVE T2DM RISK IN MHO

There are few clinical procedures for MHO individuals to prevent the high risk of incidence of T2DM, but studies have shown evidence of benefits of weight loss for MHO with the improvement of metabolic parameters and inflammatory biomarkers.

A cohort study has found that bariatric surgery could significantly achieve a great deal of total weight loss in MHO patients at follow-up[81]. Some studies have shown that MHO could achieve more weight loss than that in MUO participants after bariatric surgery[82-84], suggesting that the MHO phenotype is an independent predictor for greater body weight loss and more effective bariatric surgery in obese individuals before metabolic abnormalities appear[83]. Furthermore, cardiovascular risk factors such as blood pressure, lipid levels, and plasma glucose are improved after bariatric surgery, even when some of these levels are "normal" preoperatively[81]. Otherwise, these indexes show more improvement in metabolically unhealthy individuals[81,84]. However, Pelascini *et al*[82] failed to find significant improvements in HDL-C and plasma glucose in MHO participants, which might have resulted from their relatively small sample size and strict definition of "metabolic health" plus HOMA-IR and hs-CRP. In summary, the benefits of bariatric surgery for the MHO phenotype are considerable, potentially comparable in benefit to the unhealthier phenotype with much better weight loss[84]. However, this has only been tested and observed in MHO subjects whose BMI was ≥ 40 kg/m^2. For the majority of MHO individuals, the application of bariatric surgery is not recommended in the current clinical environment with no more solid testimonies.

For the majority of those individuals with MHO, cultivating a favorable lifestyle might be a more feasible method to achieve weight loss. Studies have demonstrated that a healthier diet with a higher proportion of fruit, vegetables, and fish and longer mealtimes (more than 10 min) in women and higher degrees of physical activity is associated with the MHO phenotype compared with the MUO phenotype[85,86]. Gomez-Huelgas *et al*[87] found that intensive lifestyle modification could induce clinically significant weight loss in MHO phenotype women, leading to the reduction of serum adipokines and inflammatory biomarkers such as hs-CRP, interleukin-6, and tumor necrosis factor-α, which play important roles in the pathological mechanism of obesity and insulin resistance.

In a prospective cohort study of the MHO population, it was found that air pollution had a significantly positive correlation with adiponectin and hs-CRP, which suggests that air pollution plays an important role in the occurrence and development of diabetes in MHO individuals[88]. It will be interesting to compare the risk of the incident in MHO with and without exposure to polluted air.

CONCLUSION

Current MHO diagnostic criteria are insufficient to exclude all obese people with the potential to develop future metabolic disorders. How to define MHO is an issue worth discussing. MHO is not absolutely "metabolically healthy" compared to MHNW with potential risks for T2DM and its related metabolic disorders. This might be explained by mechanisms such as the expansion and hypoxia of adipose tissue, increased inflammation, and decreased adiponectin concentrations in the MHO population. Liver fat accumulation is also a crucial risk factor for the incidence of T2DM in MHO. Thus, we recommend adding the intrahepatic fat content into the criteria for "metabolic health". Weight control might effectively protect the MHO individuals from the development of diabetes and its related metabolic diseases. In addition, MHO is a transitional phenotype between MHNW and MUO. It will be worthwhile to investigate the crucial factors that are responsible for the transition from MHO to MUO. The advance of multi-omics technology might help us to identify better MHO with a higher risk of developing diabetes and multiple metabolic disorders.

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World Journal of Diabetes

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World J Diabetes 2022 February 15; 13(2): 85-96

DOI: 10.4239/wjd.v13.i2.85

ISSN 1948-9358 (online)

MINIREVIEWS

Role of dipeptidyl peptidase 4 inhibitors in the new era of antidiabetic treatment

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Author contributions: All authors contributed equally to writing the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Country/Territory of origin: Greece

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

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Abstract

The last few years important changes have occurred in the field of diabetes treatment. The priority in the therapy of patients with diabetes is not glycemic control per se rather an overall management of risk factors, while individualization of glycemic target is suggested. Furthermore, regulatory authorities now require evidence of cardiovascular (CV) safety in order to approve new antidiabetic agents. The most novel drug classes, *i.e.*, sodium-glucose transporter 2 inhibitors (SGLT2-i) and some glucagon-like peptide-1 receptor agonists (GLP-1 RA), have been demonstrated to reduce major adverse CV events and, thus, have a prominent position in the therapeutic algorithm of hyperglycemia. In this context, the role of previously used hypoglycemic agents, including dipeptidyl peptidase 4 (DPP-4) inhibitors, has been modified. DPP-4 inhibitors have a favorable safety profile, do not cause hypoglycemia or weight gain and do not require dose uptitration. Furthermore, they can be administered in patients with chronic kidney disease after dose modification and elderly patients with diabetes. Still, though, they have been undermined to a third line therapeutic choice as they have not been shown to reduce CV events as is the case with SGLT2-i and GLP-1 RA. Overall, DPP-4 inhibitors appear to have a place in the management of patients with diabetes as a safe class of oral glucose lowering agents with great experience in their use.

Key Words: Cardiovascular safety; Dipeptidyl peptidase 4 inhibitors; Glucose lowering; Hypoglycemia; Therapeutic algorithm; Weight gain

Received: May 5, 2021
Peer-review started: May 5, 2021
First decision: June 16, 2021
Revised: July 29, 2021
Accepted: January 27, 2022
Article in press: January 27, 2022
Published online: February 15, 2022

P-Reviewer: Conte C, Xu PF
S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

Core Tip: Dipeptidyl peptidase 4 inhibitors have a favorable safety profile, do not frequently cause hypoglycemia and weight gain, while they may be used in patients with kidney impairment and the elderly. Despite not reducing cardiovascular events, they still have a place in the diabetes treatment algorithm in several patients.

Citation: Florentin M, Kostapanos MS, Papazafiropoulou AK. Role of dipeptidyl peptidase 4 inhibitors in the new era of antidiabetic treatment. *World J Diabetes* 2022; 13(2): 85-96
URL: https://www.wjgnet.com/1948-9358/full/v13/i2/85.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i2.85

INTRODUCTION

Diabetes mellitus (DM) is a worldwide health problem with epidemic proportions and a huge economic burden. The global prevalence of DM in 2019 was estimated to be 9.3% (463 million people) with a projection to rise to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045[1]. DM is a major cause of blindness, chronic kidney disease (CKD), stroke, lower extremity amputations and death from coronary heart disease and heart failure (HF)[2].

Until a few years ago the main focus of the management of patients with DM was the adequate or even strict glycemic control, mainly based on the fact that a glycated hemoglobin (HbA1c) of < 7% has been associated with a reduction in microvascular complications[3]. However, intensive glycemic control not only does not appear to reduce all-cause mortality and macrovascular endpoints in patients with DM type 2 (DM2), but it may increase the relative risk (RR) of severe hypoglycemia up to 30%[3,4]. Therefore, the glycemic target needs to be individualized and associated risk factors and co-morbidities be appropriately managed[5].

Another issue which emerged over a decade ago, due to concerns about agents such as rosiglitazone, is the cardiovascular (CV) safety of antidiabetic agents[6,7]. Ever since the regulatory authorities, such as the U.S. Food and Drug Administration (FDA)[8] and the European Medicines Agency (EMA)[9], require large CV outcomes trials (CVOTs) for all new treatments for DM2. Incretin-based therapies, *i.e.*, glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1 RA) and dipeptidyl peptidase 4 (DPP-4) inhibitors, and subsequent drug classes have, thus, been approved after their efficacy was established in CVOTs.

Importantly, about 6 years ago a novel class of drugs, namely sodium-glucose transporter 2 (SGLT2) inhibitors (SGLT2-i), was demonstrated to reduce major adverse CV events (MACE) and mainly hospitalizations for HF[10]. Of note, a recent meta-analysis demonstrated that SGLT2-i significantly improve CV outcomes including CV and all-cause mortality in patients with HF without excess risk of serious adverse events[11], while their capacity to slow the progression of CKD and/or albuminuria or even improve renal function has already been established[12-14].

Some GLP-1 RA were also found to decrease MACE, as well as secondary outcomes (*e.g.*, HF and progression of renal disease) in patients with established CV disease (CVD) or CKD. Furthermore, recent evidence demonstrated that these drugs reduce the risk of nonfatal stroke in patients with DM2[15].

These findings consequently changed the guidelines for the management of hyperglycemia in patients with DM2[5]. Therefore, the role of drugs which were used as second line agents (after metformin) in the therapeutic algorithm has been adjusted. DPP-4 inhibitors fall into this category. In this paper, we discuss the characteristics and CVOTs of this class of drugs as well as their current role in the therapeutic armamentarium of DM2.

MECHANISM OF ACTION AND CHARACTERISTICS OF DPP-4 INHIBITORS

In 2006 the first DPP-4 inhibitor, sitagliptin, was approved for the treatment of diabetes[16,17]. These drugs inhibit DPP-4, *i.e.*, the enzyme that degrades incretins,

subsequently prolonging their half-life[18]. Two such hormones have been identified in humans; glucose-dependent insulinotropic peptide or gastric inhibitory polypeptide (GIP) and GLP-1. The latter may achieve glucose lowering *via* various actions. Specifically, GLP-1 enhances glucose-dependent insulin secretion[19], activates insulin biosynthesis and gene transcription, thus restoring the cellular supplies of insulin for subsequent release[20], while it suppresses glucagon secretion[21,22] and food intake [23,24] and slows gastric emptying[25].

In DM2 there is a reduction in GLP-1 secretion[26], an effect which in part accounts for the impaired "incretin effect" in patients with diabetes[27]. The "incretin effect" stands for the observation that insulin response to glucose is amplified when insulin is delivered orally *vs* intravenously[28]. By inhibiting the enzyme which is responsible for the degradation of incretin hormones, *i.e.*, DPP-4, DPP-4 inhibitors prevent the proteolytic breakdown and inactivation of GLP-1 and GIP[29,30]. Typically, these drugs decrease serum DPP-4 activity by > 80%, which translates in doubling of intact, biologically active GLP-1 concentration[31] along with a significant reduction in postprandial glucose levels[31,32] and an approximately 0.8% decrease in HbA1c[33]. Importantly, DPP-4 inhibitors do not increase the risk of hypoglycemia, which is a major concern and an unfavorable prognostic factor in patients treated with antidiabetic agents. This occurs as native GLP-1, whose action is prolonged by DPP-4 inhibitors, stimulates glucose-dependent insulin secretion from pancreatic β-cells[34].

Dissimilarities in the chemical structure of the different DPP-4 inhibitors affect their pharmacokinetic properties, formulation and daily dosing (Table 1). The relatively long half-lives of sitagliptin, linagliptin and alogliptin allow for once-daily dosing. Saxagliptin, which has a short half life, may also be administered once daily due to the presence of its active metabolite, BMS-510849, which inhibits DPP-4[35-37]. In contrast, vildagliptin has a short half-life and, thus, requires twice-daily dosing[38]. As far as route of elimination is concerned, sitagliptin and alogliptin are primarily excreted renally, whereas saxagliptin undergoes both renal and hepatic clearance. In contrast, linagliptin is predominately (approximately 90%) secreted unchanged in the feces[39], while vildagliptin is metabolized *via* at least four pathways before excretion[38,40]. Regarding CKD, all DPP-4 inhibitors may be given to patients at all CKD stages in reduced doses in order to avoid increased drug exposure[38,40], with the exception of linagliptin which does not require dose modification. Furthermore, saxagliptin is contraindicated in end-stage renal disease (ESRD) and in dialysis[38] (Table 2). This agent is also prone to drug-drug interactions as it is metabolized *via* cytochrome P450 (CYP450). Hence, patients co-administered saxagliptin and CYP3A4/5 inhibitors should reduce saxagliptin dose[38,41]. Table 3 summarizes the doses which are appropriate for all stages of hepatic impairment for each DPP-4 inhibitor.

DDP-4 INHIBITORS IN CVOTS

Since over 10 years ago concerns have been raised as to the CV safety of certain antidiabetic drugs[42]. Subsequently, the FDA requires evidence of CV safety before approval of any new antidiabetic agent. In this context, no drugs that could be associated with an unacceptable level of CV risk in clinical trials would be approved for the management of DM2. Incretin-based therapies, including DDP-4 inhibitors, were the newer antidiabetic agents added to the DM2 treatment armamentarium at the time of this statement[42].

Consequently, randomized placebo-controlled clinical trials were designed to assess the CV safety of DDP-4 inhibitors. These studies mostly included high-risk patients with DM2. They had a non-inferiority design since the research question to be addressed at the time was safety rather than additional CV benefits, which were demonstrated only later with SGLT2-i and GLP-1 RA. To date, every DDP-4 inihibitor available for clinical use has been assessed in at least one of these trials (Table 4).

The trial evaluating cardiovascular outcomes with Sitagliptin (TECOS) trial included 14671 patients with DM2 with an HbA1c between 6.5 and 8.0% when treated with stable doses of one or two oral agents (*i.e.*, metformin, pioglitazone or sulfonylurea) or insulin (with or without metformin) and established CVD[43]. These patients were randomized to sitagliptin 50-100 mg/d *vs* placebo on top of standard treatment. The primary endpoint of this study was the composite of CV death, nonfatal myocardial infarction, nonfatal stroke, or hospitalization for unstable angina. This was a non-inferiority trial with upper safety boundary of 1.3 RR. During the 3 years of follow-up (median) sitagliptin was associated with mild though significant hypoglycemic effect; by lowering mean HbA1c by 0.29% points [95% confidence

Table 1 Characteristics of dipeptidyl peptidase 4 inhibitors

	Chemistry	Half-life	HbA1c reduction (%)	Metabolism	Eliminationroute
Alogliptin	Modifiedpyrimidinedione	20 h	0.6 (mean value)	Minimal	Predominantly (> 70%) renal
Linagliptin	Xanthine-based	Approxmately 12 h (effective), > 100 h (terminal)	0.5-0.7	Minimal	Predominantly biliary (< 6% renal)
Saxagliptin	Cyanopyrrolidine	2.5 h (parent), 3 h (metabolite)	0.5-1.0	Hydrolysis (cytochrome P450 3A4 or P450 3A5) to form an active metabolite	Metabolism (parent) and renal (metabolite)
Sitagliptin	β-aminoacid based	12.5 h	0.5-1.0	Minimal	Predominantly (> 80%)
Vildagliptin	Cyanopyrrolidine	Approxmately 2 h	0.9 (mean value)	Hydrolysis (cytochrome-independent) to form an inactive metabolite	Metabolism (parent) and renal (metabolite)

Table 2 Renal dosing of dipeptidyl peptidase 4 inhibitors

Renal impairment	Alogliptin	Linagliptin	Sitagliptin	Vildagliptin	Saxagliptin
Mild (eGFR > 50 mL/min)	25 mg o.d.	5 mg o.d.	100 mg o.d.	50 mg b.i.d.	5 mg o.d.
Moderate (eGFR 30-50 mL/min)	12.5 mg o.d.	5mg o.d.	50 mg o.d.	50 mg o.d.	2.5 mg o.d.
Severe (eGFR < 30 mL/min)	6.25 mg o.d.	5 mg o.d.	25 mg o.d.	50 mg o.d.	2.5 mg o.d.
ESRD	6.25 mg o.d.	5 mg o.d.	25 mg o.d.	50 mg o.d.	Contraindicated
Renal dialysis	6.25 mg o.d.	5 mg o.d.	25 mg o.d.	50 mg o.d.	Contraindicated

eGFR: Estimated glomerular filtration rate; ESRD: End-stage renal disease.

Table 3 Modification of dosing for dipeptidyl peptidase 4 inhibitors in hepatic impairment

Hepatic impairment	Alogliptin	Linagliptin	Sitagliptin	Vildagliptin	Saxagliptin
Mild	25 mg o.d.	5 mg o.d.	100 mg o.d.	Not recommended in liver disease, including AST or ALT > 3 × ULN	5 mg o.d.
Moderate	25 mg o.d.	5mg o.d.	100 mg o.d.		Can be used with caution
Severe	Not recommended	5 mg o.d.	Can be used with caution		Not recommended

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ULN: Upper limit normal.

interval (CI): -0.32 to -0.27] compared with placebo. In the intention-to-treat analysis sitagliptin was non-inferior to placebo in the primary composite endpoint [hazard ratio (HR) 0.98; 95%CI: 0.88-1.09; $P < 0.001$ for non-inferiority]. The same was relevant for all secondary CV endpoints in this trial. Interestingly, acute pancreatitis or pancreatic cancer events did not differ significantly between the sitagliptin and the placebo group. Also, sitagliptin was not associated with any excessive risk of hospitalizations for HF compared with placebo[43].

Linagliptin was evaluated in a non-inferiority multicenter randomized placebo-controlled clinical trial. The Cardiovascular And Renal Microvascular Outcome study with Linagliptin (CARMELINA) study included 6979 patients at high risk for CVD [established CVD and significant albuminuria; urine albumin creatinine ratio (UACR) > 200 mg/g] or renal disease [low estimated glomerular estimated glomerular filtration rate (eGFR) and micro- or macro-albuminuria] and suboptimal glycaemic control (baseline HbA1c 6.5%-10%)[44]. These patients were randomized to linagliptin 5 mg/d *vs* placebo. The primary composite endpoint was the time to first occurrence of CV death or nonfatal myocardial infarction or stroke. The non-inferiority margins

Table 4 Cardiovascular outcome trials with dipeptidyl peptidase 4 inhibitors

	CVOT	Comparator	Cardiovascular safety (MACE) (HR)	Risk of hospitalization for heart failure (HR)
Alogliptin	EXAMINE	Placebo	0.96	1.07
Linagliptin	CARMELINA	Placebo	1.02	0.90
	CAROLINA	Glimepiride	0.98	1.21
Saxagliptin	SAVOR-TIMI	Placebo	1.00	1.27
Sitagliptin	TECOS	Placebo	0.98	1.00

CVOT: Cardiovascular outcome trial; HR: Hazard ratio; MACE: Major adverse cardiovascular events.

were the same as in the TECOS trial.

After 2.2 years (median) follow-up the overall difference in HbA1c over the full study duration was -0.36% (95%CI: -0.42% to -0.29% based on least-square means). The primary composite outcome occurred in 5.77/100 person-years *vs* 5.63/100 person-years in the linagliptin *vs* placebo group respectively; absolute incidence rate difference was 0.13 (95%CI: -0.63 to 0.90 per 100 person-years) (HR = 1.02; 95%CI: 0.89-1.17; $P < 0.001$ for non-inferiority). Similar were the findings for the key secondary renal endpoint of composite of adjudication-confirmed ESRD, death due to renal failure, or a sustained decrease of at least 40% in eGFR from baseline. No difference in the total mortality rates was noted between groups, too. Similarly, no difference between groups was observed in the components of the key secondary renal endpoint except for progression of albuminuria which occurred less frequently in the linagliptin *vs* the placebo group: 21.4/100 person-years *vs* 24.5/100 person-years respectively; absolute incidence rate difference, -3.18; 95%CI: -5.44 to -0.92) (HR = 0.86; 95%CI: 0.78-0.95; $P = 0.003$). Regarding safety, the incidence of pancreatitis episodes and pancreatic cancer was higher in the linagliptin compared with the placebo group though the number of cases was very limited in both groups to reach safe conclusions. No statistically significant different between groups was noted in hospitalizations for HF.

The CAROLINA study was another non-inferiority study that compared linagliptin with glimepiride as an active comparator[45]. It included patients with DM2 and suboptimal glycemic control (HbA1c 6.5%-8.5%) and high CV risk. The latter was defined as the presence of established CVD or microvascular complications, the presence of multiple CV risk factors or age > 70 years. These patients were randomized to linagliptin 5 mg/d *vs* glimepiride 1-4 mg/d with investigator-led option to add other antidiabetic agents titrated to achieve sufficient glycemic control. The primary composite endpoint and the non-inferiority margins were the same as in the CARMELINA study. After 6.3 years (median) no significant difference between groups was noted in the glycemic control. Similarly, linagliptin was non-inferior to glimepiride in the primary composite endpoint which occurred in 11.8% *vs* 12.0%, respectively [HR = 0.98 (95.47% CI: 0.84-1.14); $P < 0.001$ for noninferiority; $P = 0.76$ for superiority]. The same was relevant also for the individual components of the primary endpoint[45].

Furthermore, no differences between groups were noted in overall deaths and in hospitalizations for HF. As expected, the incidence of hypoglycemic events was lower in the linagliptin than in the glimepiride group: incidence rate difference, -8.7 [95%CI: -9.4 to -8.0; HR, 0.23 (95%CI: 0.21-0.26); $P < 0.001$]. Also, more weight gain was noted in the glimepiride group, with a mean between group difference of -1.54 kg (95%CI: -1.80 to -1.28). However, no difference in fasting plasma glucose, lipids and blood pressure was noted between groups. The results of this study established the role of linagliptin as a non-inferior to sulfonylureas second-line option (after metformin) for the management of DM2[45].

Non-inferiority of alogliptin (6.25-25 mg/d adjusted according to eGFR) *vs* placebo was evaluated in 5380 high-risk participants with DM2 of the Examination of Cardiovascular Outcomes with Alogliptin *vs* Standard of Care (EXAMINE) study[46]. These patients had a recent (within 15-90 d) hospitalization for an acute coronary syndrome and suboptimal glycemic control (HbA1c 6.5%-11.0% at screening or 7.0%-11.0% if the antidiabetic regimen included insulin). The primary endpoint was the composite of CV death or nonfatal myocardial infarction or stroke and the non-inferiority margins were similar to the studies above. After 17.5 mo (median) alogliptin was associated with a mild though significant hypoglycemic effect compared with placebo; mean difference in HbA1c between groups -0.36% points (95%CI: -0.43 to -

0.28; $P < 0.001$). No significant changes between groups were noted in body weight changes or changes in lipoprotein levels. At the end of follow-up the primary endpoint occurred in similar rates in both groups: 11.3% *vs* 11.8% in the alogliptin *vs* placebo group, respectively (HR = 0.96; upper boundary of the one-sided repeated CI, 1.16; $P < 0.001$ for non-inferiority; $P = 0.32$ for superiority). No difference between groups was noted in the individual components of this endpoint or in the overall or CV mortality. No safety signal regarding the risk of acute pancreatitis or pancreatic cancer was noted in this study. Changes in eGFR throughout the study were similar between groups.

Similar was the design of the Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus-Thrombolysis in Myocardial Infarction 53 (SAVOR-TIMI53) trial[47]. This was a phase 4 randomized placebo-controlled trial including 16492 patients with DM2 with suboptimal glycemic control (6.5%-12.0%) and high CV risk (in secondary prevention or in primary prevention with multiple CV risk factors). These patients were randomized to saxagliptin 2.5-5 mg/d (adjusted based on eGFR) *vs* placebo for 2.1 years (median). The primary endpoint was the same as in the EXAMINE trial, whilst a secondary major composite endpoint of CV death, myocardial infarction, stroke, hospitalization for unstable angina, coronary revascularization, or HF was assessed too. Saxagliptin was associated with significantly reduced HbA1c compared with placebo throughout the study (difference by 0.2% points at the end of follow-up) and with more patients achieving glycemic targets. However, no significant difference between groups was noted either in the primary or in the secondary major endpoint at the end of follow-up: HR = 1.00; 95%CI: 0.89-1.12; $P = 0.99$ for superiority; $P < 0.001$ for non-inferiority for the primary endpoint and HR = 1.02; 95%CI: 0.94-1.11; $P = 0.66$ for the secondary endpoint. Interestingly, among the individual components of these endpoints saxagliptin was associated with an increased risk of hospitalization for HF compared with placebo (HR = 1.27; 95%CI: 1.07-1.51; $P = 0.007$). As mentioned above no similar signal was identified with sitagliptin and linagliptin in the TECOS and CARMELINA trial, respectively.

This matter is of particular significance since worsening of HF has been associated with excessive mortality in patients with DM2. To further assess this question the Vildagliptin in Ventricular Dysfunction Diabetes (VIVIDD) trial included 254 patients with symptomatic HF [New York Heart Association (NYHA) class II and III] with reduced left ventricular ejection fraction (LVEF < 40%) and a HbA1c of 6.5%-10%[48]. These patients were randomized to vildagliptin 50 mg twice daily *vs* placebo for 52 wk. Vildagliptin was non-inferior to placebo in mean changes of LVEF assessed after ≥ 22 wk on treatment (adjusted mean change 4.95% ± 1.25% *vs* 4.33% ± 1.23% in the vildagliptin *vs* placebo group, respectively). This was not accompanied by any differences between the 2 groups regarding other HF outcomes, including NYHA classification status and hospitalizations for HF.

However, vildagliptin was associated with significant increases in the end-diastolic LV volume as well as a non-significant trend to increased end-systolic one. The latter could be attributed to pre-treatment differences between groups in this regard. Namely, mean baseline end-diastolic volumes and brain natriuretic peptide were higher in the vildagliptin than in the placebo group. Hence, patients randomized to vildagliptin may have been more susceptible to such changes. However, the clinical relevance of this finding was uncertain and was not accompanied with any worse HF outcomes.

Overall, the large-scale randomized placebo-controlled trials with DDP-4 inhibitors established their CV and overall safety for the management of high-risk patients with DM2. However, no evidence of superiority was demonstrated in CV outcomes as compared with controls or sulfonylurea treatment. To date, there are no published head-to-head comparison CVOTs between DDP-4 inhibitors and antidiabetic drugs with established CV efficacy such as SGLT2-i or GLP1-RA. Overall, the modest hypoglycemic effects alongside the neutral effect of DDP-4 inhibitors on the lipid profile, blood pressure and body weight make DDP-4 inhibitors less promising for CVD prevention compared with the SGLT2-i and GLP-1 RA[49]. Indeed, in a network meta-analysis (236 trials; 176,310 patients) the use of SGLT2-i or GLP1-RA was associated with lower mortality compared with DPP-4 inhibitors or placebo or no treatment. Treatment with DPP-4 inhibitors was not associated with lower mortality compared with placebo or no treatment[50].

SAFETY

No safety signals were identified in the aforementioned clinical trials in the risk of

acute pancreatitis or pancreatic cancer. These two clinical entities were regarded important safety issues until up to a few years ago, as there were several relevant reports and signals from clinical studies with these drugs[51]. However, a recent meta-analysis of randomized controlled trials demonstrated that the available data do not support an association of DPP-4 inhibitors with pancreatitis or pancreatic cancer. We should note that the evidence regarding pancreatic cancer is more limited and, thus, insufficient to draw definitive conclusions[52]. The excess of hospitalizations for HF associated with saxagliptin in the SAVOR-TIMI53 trial was not observed with the other DDP-4 inhibitors in CVOTs except a non-significant rise in the EXAMINE trial with alogliptin. In this context, regulatory authorities have added a warning in the labels of saxagliptin and alogliptin for the increased risk of HF[53]. The results of the VIVIDD study were reassuring as for the drug class. However, this matter should be investigated more in future longitudinal studies as the relatively short follow-up of these CVOTs may not be sufficient to detect a relevant safety signal.

Furthermore, as previously mentioned, this drug class does not increase the risk of hypoglycemia and is neutral in terms of weight gain, two issues important for patients with DM2, while other side effects are minor and reversible (*e.g.*, gastrointestinal adverse effects, flu-like symptoms).

CURRENT USE OF DPP-4 INHIBITORS

DPP-4 inhibitors were the first therapeutic choice after metformin initiation only up to a few years ago as they improve glycemic control without producing hypoglycemia or weight gain[54]. However, the inability to show a beneficial effect in morbidity and mortality as well as the significant findings of the large-scale CVOTs of the newer antidiabetic agents (*i.e.*, SGLT2-i and GLP-1 RA) have moved DPP-4 inhibitors lower in the algorithm of hyperglycemia management[5]. The above-mentioned change in the prescription of antidiabetic agents during the last years is reflected by the results of a recent study in Greece[55]. The percentage of patients treated with a DPP-4 inhibitor, a GLP-1 RA or a SGLT2-i in 2018 was 43.4%, 18.5% and 16.5%, respectively[55].

However, previous studies reflect the large use of DPP-4 inhibitors as a second choice of antidiabetic agents almost a decade ago. A large epidemiology study in the United States in a cohort of patients aged 18 years to 100 years who were newly initiated on oral hypoglycemic monotherapy between January 1, 2006, and December 31, 2008, showed that the greatest relative change for the study period was observed for the DPP-4 inhibitors, increasing from 0.4% to 7.3% or 0.15% per month[56]. Of note, during the period that the study was conducted GLP-1 RA and SGLT2-i were not available and, therefore, were not included in the analysis. The same pattern was observed in a study in Germany in elderly patients with an initial diagnosis of DM2 between January 2011 and December 2015, where the use of DPP-4 inhibitors raised from 13.4% to 19.8% during the study period[57]. The results of the study showed that DPP-4 inhibitors might be preferred over other drugs due to the good safety profile in elderly patients with DM2. At this point we should mention that there is lack of evidence regarding the trends of prescription of DPP-4 inhibitors. Another rather important issue is that there are large differences in prescription patterns, suggesting that the screening and management of DM2 varies among different countries.

THE PLACE OF DPP-4 INHIBITORS IN THE THERAPEUTIC ALGORITHM OF HYPERGLYCEMIA

In general, DPP-4 inhibitors cause a clinically meaningful reduction in blood glucose, have a low risk of hypoglycemia and a neutral effect on body weight, while their safety profile is overall favorable. They are also easy to use, requiring no dose titration and can be taken at any time of day regardless of meal times. Furthermore, DPP-4 inhibitors exhibit non-glycemic favorable effects including reductions in systolic blood pressure, total cholesterol and triglycerides, as well as improvement in β-cell function [35]. For the above reasons, until recently, they were a safe choice for the up titration of antidiabetic therapy after metformin. However, the large CVOTs with the newest agents, namely GLP-1 RA and SGLT2-i, have changed the treatment algorithm as well as the selection of DPP-4 inhibitors as a second-line add-on therapy to metformin[5].

DPP-4 inhibitors still have a place in the treatment of certain patients, such as those who take many drugs due to longstanding DM2 and have multiple co-morbidities, as

well as in those with renal impairment, where other anti-diabetic medications might be contraindicated. The frail elderly population may also benefit due to the low risk of hypoglycemia with DPP-4 inhibitors. Post-hoc analysis of the SAVOR-TIMI 53 data established the safety and efficacy of saxagliptin in the elderly[58], an observation that has been confirmed by other studies of DPP-4 inhibitors in this patient population[59, 60]. We should stress that saxagliptin is contraindicated in patients with HF due to the increased risk of hospitalizations for HF associated with its use[47].

Patients with advanced renal failure have fewer options of glucose lowering agents and often resort to treatment with complicated insulin regimens facing their accompanying increased hypoglycemia risk. Linagliptin might be a good choice as initial therapy in a patient with CKD at risk for hypoglycemia, while other DPP-4 inhibitors might be used with proper dose adjustment in these patients[38,39]. More recently, renoprotection was suggested as another beneficial property of DPP-4 inhibitors[36], which may be of clinical importance as diabetic nephropathy is a major complication of DM. Experimental data suggest that the modulation of innate immunity and inflammation are probably involved in these kidney-protective effects. The degradation of DPP-4, which is known to be expressed on the cell membrane of many types of cells including immune cells, as well as of several chemokines and cytokines[36], the attenuation of oxidative stress, fibrosis and cellular apoptosis in the kidney[37] are plausible underlying mechanisms.

According to recent guidelines, in patients with DM2 and established atherosclerotic CVD a GLP-1 RA or an SGLT2-i with proven CV safety (*i.e.*, it has label indication of reducing CVD events) should be preferably used. In patients with HF or CKD an SGLT2-i should be used due to the beneficial effects of these drugs in CVOTs, unless they are contraindicated (according to GFR levels); then a GLP-1 RA should be used [61].

When the therapeutic goals are not achieved with the previous antidiabetic agents, a combination with a DPP-4 inhibitor is recommended as a possible third-line therapy. The triple therapy of metformin with a DPP-4 and an SGLT2-i has a very low risk of hypoglycemia, leads to a further reduction in HbA1c, followed by weight loss and a reduction of blood pressure secondary to SGLT2-i administration[62-64]. Moreover, the dual effects of DPP4-i on α-cells and β-cells of the pancreas may combine well with the pancreatic islet-independent action of SGLT2-i.

DPP-4 inhibitors still remain a reasonable second-line add-on therapy to metformin, especially in individuals at high risk for hypoglycemia (*i.e.*, elderly) or when an oral regimen is preferred. DPP-4 inhibitors can also be combined with insulin therapy. The combination of basal insulin with a DPP-4 inhibitor is a practical treatment option without the need for multiple injections and glucose self-measurements for the adjustments of insulin[61].

CONCLUSION

Despite the establishment of SGLT2-i and GLP-1 RA as a second-line therapy in current diabetes treatment algorithms, DPP-4 inhibitors still remain a useful tool for the management of patients with diabetes. Furthermore, the lack of evidence with SGLT2-i and GLP-1 RA in elderly patients with diabetes as well as the contraindication of SGLT2-i in patients with CKD grade 3A and lower, make DPP-4 inhibitors a safe choice in such populations. Concluding, DPP-4 inhibitors still appear to have a place in the management of patients with DM2 as a safe class of oral glucose lowering agents with great experience in their use.

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 February 15; 13(2): 97-109

DOI: 10.4239/wjd.v13.i2.97

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Clinical and Translational Research

Altered spontaneous brain activity patterns in patients with diabetic retinopathy using amplitude of low-frequency fluctuation

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Institutional review board statement: The research methods and protocols were approved by the Medical Ethics Committee of the First Affiliated Hospital of

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Abstract

BACKGROUND

Diabetes mellitus is a metabolic disorder characterized by prolonged elevation of blood glucose due to various causes. Currently, the relationship between diabetic retinopathy (DR) and altered connectivity of brain function is unclear.

AIM

To investigate the relationship between this brain activity and clinical manifestations and behaviors of DR patients by using the amplitude of low-frequency fluctuation (ALFF) technique.

METHODS

Twenty-four DR patients and 24 healthy controls (HCs) matched for age and gender were enrolled. We measured and recorded average ALFF values of DR patients and HCs and then classified them using receiver operating characteristic (ROC) curves.

RESULTS

ALFF values of both left and right posterior cerebellar lobe and right anterior cingulate gyrus were remarkably higher in the DR patients than in the HCs; however, DR patients had lower values in the bilateral calcarine area. ROC curve analysis of different brain regions demonstrated high accuracy in the area under

the curve analysis. There was no significant relationship between mean ALFF values for different regions and clinical presentations in DR patients. Neuronal synchronization abnormalities in some brain regions of DR patients were associated with cognitive and visual disorders.

CONCLUSION

Abnormal spontaneous brain activity was observed in many areas of DR patients' brains, which may suggest a possible link between clinical manifestations and behaviors in DR patients.

Key Words: Amplitude of low-frequency fluctuation; Functional magnetic resonance imaging; Diabetic retinopathy; Resting-state; Diabetes mellitus; Spontaneous activity

Core Tip: We found that patients with diabetic retinopathy (DR) may have multiple low-frequency amplitude frequency changes in the brain, and the generation of this change may be related to the alteration of patients' visual cortex and anxiety, which may help us to explore the pathological mechanism and disease progression in DR patients.

Citation: Shi WQ, Zhang MX, Tang LY, Ye L, Zhang YQ, Lin Q, Li B, Shao Y, Yu Y. Altered spontaneous brain activity patterns in patients with diabetic retinopathy using amplitude of low-frequency fluctuation. *World J Diabetes* 2022; 13(2): 97-109
URL: https://www.wjgnet.com/1948-9358/full/v13/i2/97.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i2.97

INTRODUCTION

Chronic complications of diabetes are the main causes of death and disability with diabetes, and its morbidity may be related to many factors, among which diabetic retinopathy (DR) is the main cause of blindness and visual impairment[1]. The incidence of DR is increasing worldwide mainly because of the longevity of diabetes patients[2]. A large proportion of adults who are over the age of 40 are affected by DR. Early manifestations of DR are mainly aneurysms, bleeding spots, hard exudation, cotton buds, venous beading, intravascular microvascular abnormalities, and macular edema[3]. According to the presence of retinal neovascularization, we can divide DR into proliferative DR (PDR) and non-proliferative DR (NPDR). In PDR, retinal damage stimulates neovascular growth. Neovascular growth is detrimental to the retina, which can cause fibrosis and even retinal detachment. Neovascularization can also enter the vitreous, which will lead to vitreous hemorrhage[4] (Figure 1). Recently, there has been increasing evidence that similar microvascular lesions happen in the brains of diabetics. Autopsy results in patients with chronic diabetes have shown that their brains developed a severe microvascular disease and neurological disease[5]. Pearce *et al*[6] pointed out that a diagnosis of DR suggests an increased risk of brain parenchymal disease in diabetic patients. These diabetic vascular and neurological complications interact for a long time, and we believe that the effects of diabetic complications on the brain's microvasculature are often overlooked. The previous gold standard for a DR diagnosis relied mainly on fundus fluorescence imaging, but it is not suitable for people with skin test allergies and poor liver and kidney function. However, there has been little analysis of changes in brain function in DR and its relationship to clinical manifestations in the eye to date. We hypothesized that the presence of DR may suggest central damage to the brain neural network caused by the existence of a microvascular system, while central damage in the brain neural network is an early predictor of DR.

Functional magnetic resonance imaging (fMRI) makes it possible to observe specific differences in the brain neural network[7] and studying these changes can enhance our knowledge of diseases. Thus, fMRI neuroimaging is of great interest for exploring the function of the central nervous system, considering it can monitor neural activity in

Nanchang University (Ethics approval number: 2017035), following the principles of the Declaration of Helsinki. Notify all subjects of the purpose, content and potential risks of the study, and provide written informed consent.

Clinical trial registration statement: This study is registered at Medical Ethics Comitee of the First Affiliated Hospital of Nanchang University trial registry. The registration identification number is JX2017035.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: This was not an industry supported study. The authors report no conflicts of interest in this work.

Data sharing statement: The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Country/Territory of origin: China

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 1, 2021
Peer-review started: January 1, 2021
First decision: July 28, 2021
Revised: August 10, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: February 15, 2022

P-Reviewer: Kotzalidis GD
S-Editor: Gao CC
L-Editor: A
P-Editor: Wang LYT

the brain and can also provide some new explanations for the pathophysiological mechanism and pathogenesis of disease[8]. Resting-state fMRI (rs-fMRI) is a functional magnetic resonance technique used in resting-state functional network research. It explores the human nervous system through magnetic resonance imaging, which is simpler than task-state fMRI[9]. By combining electroneurophysiological records with fMRI, studies have demonstrated that low frequency (0.0-0.08 Hz) fluctuations (LFF) in blood oxygen level dependent (BOLD) fMRI signals are closely associated with spontaneous neuronal activity[10]. ALFF is one of the methods used to evaluate fMRI analysis of resting brain activity[11,12]. It reflects the intensity of local spontaneous brain activity at rest and brain endogenous/background neurophysiological processes. We have used the ALFF method to assess the neurological status and brain changes in some patients with eye diseases; for example, optic neuritis[13], glaucoma and strabismus[14,15], Parkinson's disease[16], in our previous studies.

MATERIALS AND METHODS

Subjects

We randomly selected 24 DR patients who visited the First Affiliated Hospital of Nanchang University, Nanchang, China. All the subjects met the following inclusion criteria: (1) Diagnosis of type 2 diabetes; (2) Diagnosis of PDR; (3) Fasting blood glucose controlled at approximately 7-10 mmol/L and blood glucose after a meal at 10-14 mmol/L; (4) No abnormality of the cerebral parenchyma on cranial MRI; (5) Without other ocular diseases bilaterally (*e.g.*, retinal detachment, glaucoma, amblyopia, ocular trauma, optic nerve disease); (6) Right-handed; and (7) Untreated PDR. The exclusion criteria were: (1) Smokers; (2) No other eye diseases; (3) Pregnant or lactating women; (4) Other diabetes complications; (5) Congenital systemic disease; (6) Mental illness (such as depression, memory impairment, cognitive impairment, and schizophrenia); and (7) Cerebral infarction diseases or cerebral vascular malformations. Twenty-four healthy controls (HCs) matched in age, educational status, and sex were enrolled. Both the DR group and HCs met the following criteria: (1) MRI showed no obvious damage or deformity of the brain parenchyma; (2) No history of brain infarction, cardiovascular disease or cerebral hemorrhage; (3) No evidence of drug or alcohol addiction; and (4) Were able to tolerate an MRI examination.

This study was conformed with the Declaration of Helsinki and had formal approval from the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University. All the volunteers signed informed consent forms and were allowed to ask questions after learning about the purpose, content, and potential risks of this research.

Diagnostic criteria

The International Council of Ophthalmology in Sydney defined the international classification standard of DR in 2002, and the details are as follows: (1) In the early stage of the disease, after mydriasis, ophthalmoscopy revealed diffuse microaneurysms and small petechia in the posterior pole of the retina; some patients exhibited white or yellow exudate with complaints of blurry vision; (2) Retinopathy was found in the fundus under fundus angiography machine; (3) Using fundus fluorescein angiography, the number of microadenomas in the fundus was obviously increased and were beyond the results of fundus microscopy and there was capillary dilation around the retina, increased permeability, and increased abnormalities such as bleeding or neovascularization[17]. A patient with any of these symptoms was diagnosed as DR.

MRI parameters

In this research, we performed MRI scans using 3-Tesla MRI scanners (Siemens, Munich, Germany). All participants were asked to close their eyes and maintain natural breathing until the end of the scan. We applied a gradient echo sequence of pulses of the 3D variation to obtain function data, and the parameters were as follows: 176 structural images (acquisition matrix = 256 × 256, field of view = 250 mm × 250 mm, TR = 2 s, TE = 2.26 ms, cycle time = 1900 ms, thickness = 1.0 mm, gap = 0.5 mm, flip angle = 9°). We acquired 240 functional images in total (acquisition matrix = 64 × 64, field of view = 220 mm × 220 mm, thickness = 4.0 mm, gap = 1.2 mm, cycle time = 2000 ms, echo time = 30 ms, flip angle = 90°, 29 axial). A typical scan took 15 min to complete.

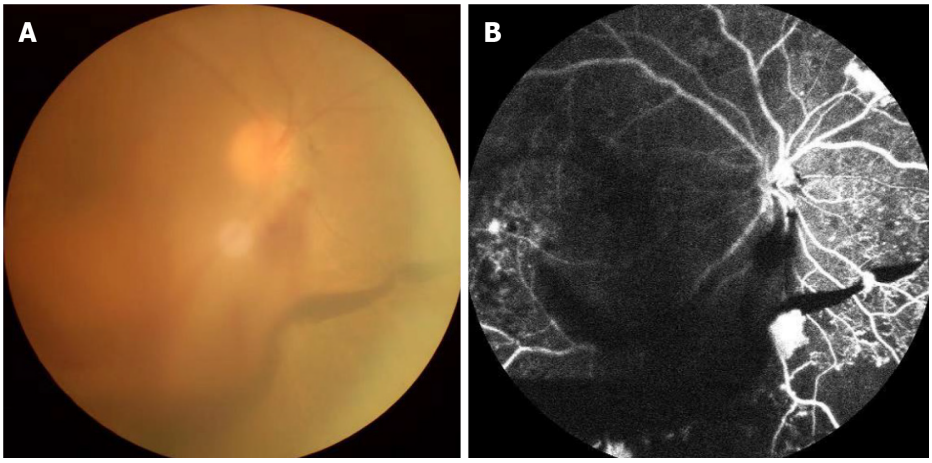

Figure 1 Example of diabetic retinopathy shown on fundus photography and fluorescein angiography.

fMRI data analysis

Our previous reports described how to analyze fMRI data. First, we used MRIcro software (www.mricro.com) to expel broken data. During magnetization equilibration, the first ten time points were discarded. Data Processing Assistant for the advanced edition of Resting-State fMRI (DPARSFA 4.0, http://rfmri.org/DPARSF) software was used for the form conversion of digital imaging communications in medicine (DICM), the correction of head motion, slice timing, realignment, spatial normalization, full-width smoothing with a Gaussian kernel of 6 mm × 6 mm × 6 mm at half-maximum, detrending, and nuisance covariates regression, based on the rs-fMRI data analysis toolkit (REST, http://www.restfmri.net). The other images were corrected time differences and micromotions during scanning. During the fMRI examination, subjects were excluded if they had more than three degrees of motion or if the maximum excursion in the x, y or z direction exceeded 3 mm.

We used linear regression analysis to remove spurious covariates and their time derivatives from various other sources, including the signal from the region of interest (ROI) to the central white matter region[18]. It should be noted that, in this study, the data showed that the global signal did not shrink, as was the case in our former study [19,20], which may have been caused by the elimination of global signals during preprocessing of the data at rest[21,22]. The fMRI images were unified to the Montreal Neurological Institute spatial standard using a standard echoplanar imaging template after correction for head motion, while the images were resampled to a resolution of 3 mm × 3 mm × 3 mm. To reduce the effect of diversity between participants, we divided the ALFF of each voxel by the average whole-brain ALFF value for each subject.

Brain-behavior correlation analysis

According to the results of ALFF, REST software was applied to divide different brain areas of these groups into areas of interest. The mean ALFF values in each region of interest were obtained by calculating the average ALFF values of all voxels. Using GraphPad Prism 9.0 (Graph Pad Software Inc., San Diego, CA, United States), the correlation between the average ALFF values of multiple brain regions in the DR group and the clinical data was evaluated ($P < 0.05$).

Statistical analysis

We used the SPSS version 22.0 (IBM Corporation, Armonk, NY, United States) with an independent samples *t*-test to analyze the cumulative clinical variables between HCs and the DR group. A P value < 0.05 showed statistical significance. We compared functional data with a two-sample *t*-test using REST software. Through Gaussian random field theory, the statistical thresholds for multiple comprehensively compared voxel levels were set as $P < 0.001$. Alphasim was corrected for cluster sizes > 30 voxels at a $P < 0.01$ level.

RESULTS

Demographics and behavioral results

No statistically significant differences were found between the two groups in weight (P = 0.982) or age (P = 0.975). The mean ± SD of DR duration was 253.18 ± 76.22 d (Table 1).

ALFF differences

Compared with the results in the HCs, the ALFF values of the DR patients were remarkably lower in the bilateral calcarine fissures, but higher in the left and right posterior lobes of the cerebellum as well as the right anterior cingulate gyrus (Figures 2 and 3 and Table 2).

Receiver operating characteristic curves

Receiver operating characteristic (ROC) curves were applied to analyze the mean ALFF values of different cerebral areas. The diagnosis rate was displayed in the area under the curve (AUC). The AUCs of ALFF values for different cerebral areas were as follows: Right anterior cingulate gyrus (0.080, P < 0.001), left posterior lobe of the cerebellum (0.938, P < 0.001), right posterior lobe of the cerebellum (0.947, P < 0.001; Figure 4A), and the bilateral calcarine fissures (0.893, P < 0.001; Figure 4B).

Correlation analysis

There was a positive correlation between the best-corrected visual acuity (BCVA) values of the affected eyes in the DR group and bilateral calcarine signal values (r = 0.938, P = 0.001). Figure 5 shows the specific details.

DISCUSSION

DR comprises microvessel damage, which is caused by diabetes mellitus. It is one of the most important causes of visual impairment in young adults[23]. DR is found in 33% of the diabetic patients in the world and is considered an increased risk for systemic vascular complications[24]. The retinal anatomy, physiology and embryological features are similar to cerebral small blood vessels of the brain[25]. Research has shown fundus arteriosclerosis has a positive correlation with cerebral atherosclerosis (P < 0.01)[26], and many diabetes neuroimaging markers of brain abnormalities have a relationship with microvascular disease. Previous studies have shown that the retinal pathological changes in the microcirculation (for example, the Gunn arteriovenous phenomenon, microaneurysms, soft exudate, and retinal hemorrhages) may be associated with markers of cerebral microvascular disease[27].

To our knowledge, this is the first study designed to explore spontaneous cerebral activity changes in DR patients using the ALFF method. Compared with the values in the HCs, the DR patients showed significantly lower ALFF values in the bilateral calcarine fissures, but higher values in the left and right posterior lobes of the cerebellum and the right anterior cingulate gyrus (Figure 6). The ALFF method has already been successfully applied in several eye diseases (Table 3) and it is expected that the application has good prospects for the future.

Analysis of the decreased ALFF values of DR patients

The calcium troponin crack is a deep groove on the inside surface of the occipital lobe. The upper and lower parts of the sulcus are the main cortical projection areas of vision. Impulses from the upper retina are transmitted above the sulcus, and visual information from the lower retina is projected below the sulcus. Impulses from the macular area are transmitted above and below the posterior third of the sulcus. This structure is an anatomical marker or landmark.

Previous reports[28] have shown that diabetes affects the microvascular system and leads to cerebral small vascular disease (SVD; Figure 7). The underlying mechanism of visual impairment in DR may involve a compromised arterial blood supply to the visual region that results from SVD. As fMRI has shown, bilateral activation of the visual cortex and eyeball movement can be caused by stimulation of the visual system [29]. Previous studies[30] have shown that prolonged poor glycemic control manifests with basement membrane thickening, tight junction disruption, and pericyte loss, leading to dysregulation of the vascular tone and endothelial cell proliferation, which can lead to microaneurysm formation and ultimately spot and speckle hemorrhages.

Table 1 Demographics and clinical measurements by groups

Condition	DR	HC	t	*P* value
Male/female	10/14	10/14	N/A	> 0.99
Age (yr)	54.31 ± 5.96	53.08 ± 5.33	0.093	0.975
Weight (kg)	53.67 ± 9.64	59.86 ± 9.93	0.095	0.982
Handedness	24R	24R	N/A	> 0.99
Duration of DR (d)	253.18 ± 76.22	N/A	N/A	N/A
Best-corrected VA-right eye	0.19 ± 0.08	1.12 ± 0.36	-0.883	0.009
Best-corrected VA-left eye	0.22 ± 0.09	1.09 ± 0.42	-0.743	0.014
IOP-R (mmHG)	15.63 ± 2.93	17.56 ± 2.74	0.092	0.815
IOP-L (mmHG)	16.13 ± 2.32	16.54 ± 2.63	0.088	0.843

$P < 0.05$ independent *t*-tests comparing two groups. DR: Diabetic retinopathy; HC: Healthy control; N/A: Not applicable; R: Right; IOP: Intraocular pressure.

Table 2 Brain areas with significantly different amplitude of low-frequency fluctuation values between groups

Condition	L/R/B	Brain regions	MNI coordinates			BA	Peak voxels	*t* value	*P* value
			X	Y	Z				
DRs > HCs									
1	L	Cerebellum posterior lobe	-27	-72	-36	/	238	5.4338	< 0.001
2	R	Cerebellum posterior lobe	33	-57	-42	/	111	4.6875	< 0.001
3	R	Anterior cingulate	9	48	3	32	32	-4.4176	< 0.001
DRs < HCs									
1	B	Calcarine	3	-66	21	/	46	-4.3494	< 0.001

$P < 0.05$, $P < 0.001$, independent *t*-test, *P* values between diabetic retinopathies and healthy controls. BA: Brodmann area; DR: Diabetic retinopathy; HC: Healthy control; MNI: Montreal Neurological Institute; L: Left; R: Right; B: Bilateral.

In this study, we found that there were decreased ALFF values bilaterally in the calcarine fissures of DR patients, which may be related to the impaired vision in these patients. We examined BCVA in both eyes of the patients and found that patients with DR had significantly lower BCVA compared to HCs ($P < 0.05$; Table 1). Our study observed that there was a positive correlation between the BCVA values of the eyes of the DR group and the signal values of the bilateral calcarine ($r = 0.938$, $P = 0.001$). It may be speculated that the decreased signal values of the bilateral calcarine fissures may reflect damage to visual processing in the patients.

Analysis of the increased ALFF values in DR

Traditionally, the cerebellar function is considered to involve physical balance and motor coordination[31]. However, because of the development and application of neuroimaging technology in recent years, we have a better understanding of the specific effect of the cerebellum, especially its posterior lobe, in emotional processing [32].

The cerebellum's posterior lobe is located between the primary fissure and the posterolateral fissure. In earlier studies, lesions of the posterior lobe could cause severe damage to spatial memory, emotional regulation, and executive functions[33,34]. Nitschke *et al*[35] showed that the cerebellum is associated with the movement of the eyeballs. Kresyun[36] found that using a transcranial magnetic to stimulate the cerebellum of DR patients helped with the recovery of the retina's ability to respond to light stress. Previous studies employing positron emission tomography found that patients with social anxiety had abnormal cerebellum signals with increasing cerebral

Table 3 Amplitude of low-frequency fluctuation method applied in ophthalmological diseases

Ref.	Disease	Brain areas	
		PG > HG	PG < HG
Guo *et al*[46], 2010	High myopia	LCN; Thalamus; Cuneus	LOL; BFL; RIPL; RPL; RMTL
Li *et al*[14], 2014	Glaucoma	RPG	LPG; BMFG; BSFG; RP; RAG
Liu *et al*[13], 2018	Optic neuritis	LCPL; RCPL; RITG; LPG; RFG; LFG; LCF; LIPL; LC	RCPL; RCAL; RP; RIFG; RI;LMFG; LSTG; RSG; BAC;BP;RIPL
Xi *et al*[15], 2010	Strabismus	BT	POS
Liang *et al*[48], 2016	Amblyopia	BC; LMOG; LPG	BPC
Tan *et al*[47], 2016	Open-globe injury	LC; LMCC; BP	
Pan *et al*[49], 2018	Acute eye pain	LPG; RPG; LC	LPG; RPG; LP

HG: Healthy group; PG: Patient group; LOL: Left occipital lobe; BFL: Bilateral frontal lobe; RIPL: Right inferior parietal lobule; RPL: Right parietal lobe; RMTL: Right middle temporal lobe; LCN: Left caudate nucleus; LPG: Left precentral gyrus; BMFG: Bilateral middle frontal gyrus; BSFG: Bilateral superior frontal gyrus; RP: Right precuneus; RAG: Right angular gyrus; RPG: Right precentral gyrus; LCPL: Left cerebellum posterior lobe; RCPL: Right cerebellum posterior lobe; RITG: Right inferior temporal gyrus; LPG: Left parahippocampal gyrus; RFG: Right fusiform gyrus; LFG: Left fusiform gyrus; LCF: Left calcarine fissure; LIPL: Left inferior parietal lobule; LC: Left cuneus; RCAL: Right cerebellum anterior lobe; RP: Right putamen; RIFG: Right inferior frontal gyrus; RI: Right insula; LMFG: Left medial frontal gyrus; LSTG: Left superior temporal gyrus; RSG: Right supramarginal gyrus; BAC: Bilateral anterior cingulate; BP: Bilateral precuneus; BCPL: Bilateral cerebellum posterior lobe; LAG: Left angular gyrus; BMG: Bilateral medialfrontal gyrus; BC: Bilateral calcarine; LMOG: Left middle occipital gyrus; LPG: Left postcentral gyrus; BPC: Bilateral precuneus cortex; LMCC: Left middle cingulum cortex; BP: Bilateral precuneus; RPG: Right parahippocampal gyrus; LPG: Left precentral gyrus; LC: Left caudate; RPG: Right precentral/postcentral gyrus; LP: Left precuneus; BT: Bilateral thalamus; POS: Parieto-occipital sulcus.

blood flow, which were illustrative. All these studies showed that abnormal activity in the cerebellum was related to anxiety[37,38]. In this study, we found an increase in the ALFF values of the left and right posterior cerebellar lobe. While we cannot prove that DR patients have anxiety, we can propose the hypothesis that anxiety may occur in DR patients with visual and even cognitive disorders.

The anterior cingulate cortex (ACC) is a vital part of the limbic system of the brain. It has extensive and numerous fibrous connections to the cortex and subcortical structures and is involved in the regulation of emotional and motor functions[39]. In a previous study, researchers observed structural defects in the ACC in many depressed patients[40]. Yu *et al*[41] evaluated diabetic patients using the SF-36 Health-Related Quality of life (HRQL) and anxiety disorders, and they found that there was a statistically significant difference in impaired HRQL (SF-36 summary score) in DR patients compared to the control group ($P < 0.05$). Clinically, DR patients may also have serious psychosocial problems[42]. Depression is not uncommon in DR patients and it has a negative impact on their condition. However, in recent years, an increasing amount of evidence from electrophysiology[43], functional imaging[44], and behavioral studies [45] have shown that the ACC is closely related to the management of pain. The ACC can be activated by nociceptive and contextual stimuli, and it can participate in pain management, especially affective pain. The neural mechanisms of the ACC's involvement in effective pain have not been clarified. The specific neural mechanism remains to be studied in the future.

CONCLUSION

This study demonstrated that there are some abnormal spontaneous brain activities in DR patients. The findings using these new techniques offer information that may help to explain the nerve mechanisms underlying the clinical manifestations of DR patients and contribute to improved clinical diagnoses.

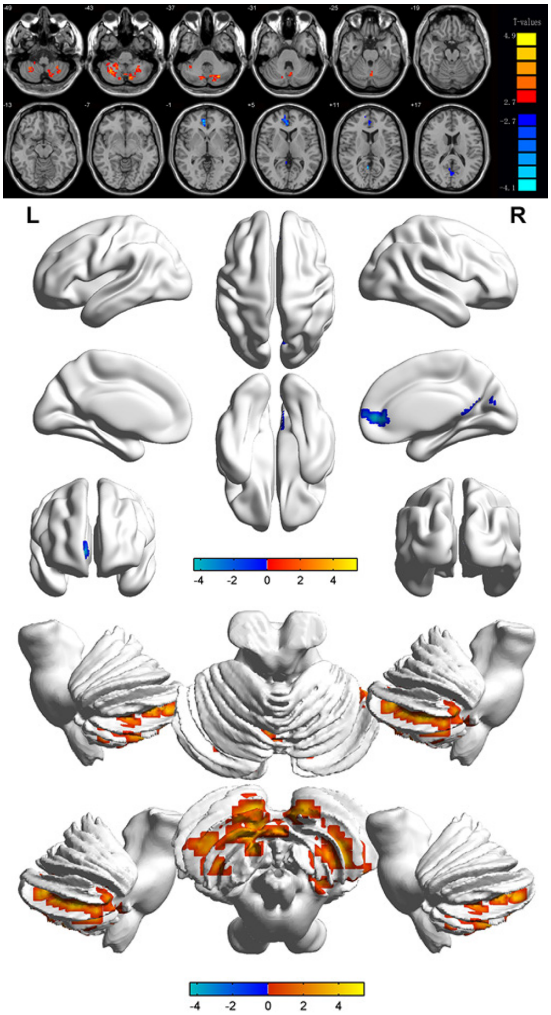

Figure 2 Significant differences in spontaneous brain activity between the diabetic retinopathy group and healthy controls. The different brain regions were observed in the left cerebellum posterior lobe, right cerebellum posterior lobe, right anterior cingulate and bilateral calcarine. The red areas denote higher amplitude of low-frequency fluctuation (ALFF) brain regions, and the blue areas denote lower ALFF brain regions [$P < 0.001$ for multiple comparisons using Gaussian random field theory (z.2.3, $P < 0.01$, cluster > 30 voxels, Alphasim corrected)].

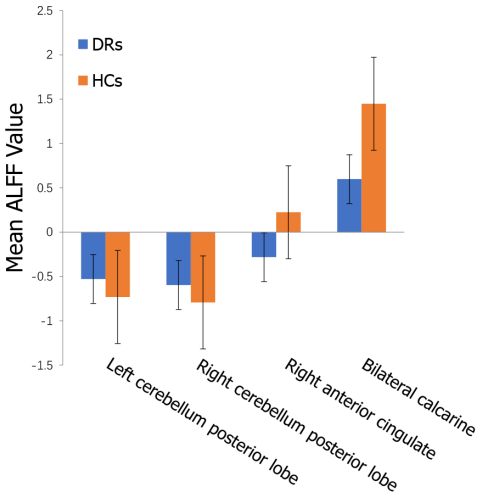

Figure 3 Means of altered spontaneous brain activity between the diabetic retinopathy group and healthy controls. DR: Diabetic retinopathy; HCs: Healthy controls; ALFF: Amplitude of low-frequency fluctuation.

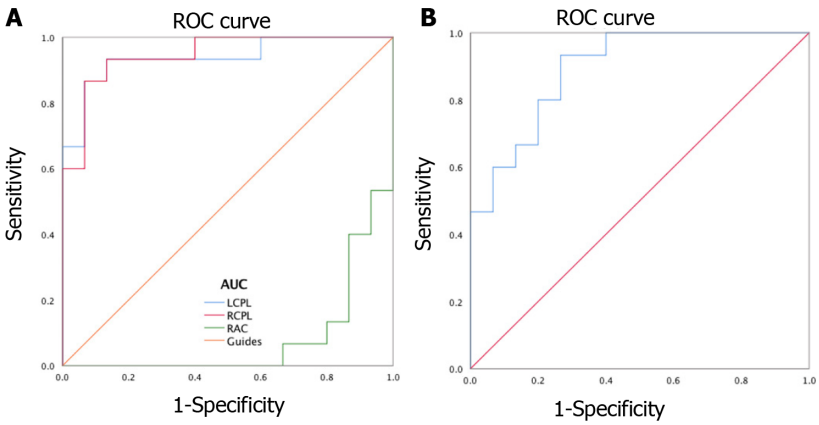

Figure 4 Receiver operating characteristic curve analysis of the mean amplitude of low-frequency fluctuation values for altered brain regions. A: The area under the ROC curve were 0.080, [$P < 0.001$; 95% confidence interval (CI): 0.000 to 0.177] for RAC, LCPL 0.938 ($P < 0.001$; 95%CI: 0.849 to 1.000), RCPL 0.947 ($P < 0.001$; 95%CI: 0.871 to 1.000) [diabetic retinopathies (DRs) > healthy controls (HCs)]; B: The area under the ROC curve were 0.893 ($P < 0.001$; 95%CI: 0.782 to 1.000) for BC (DRs < HCs). ROC: Receiver operating characteristic; AUC: Area under the curve; LCPL: Left cerebellum posterior lobe; RCPL: Right cerebellum posterior lobe; RAC: Right anterior cingulate.

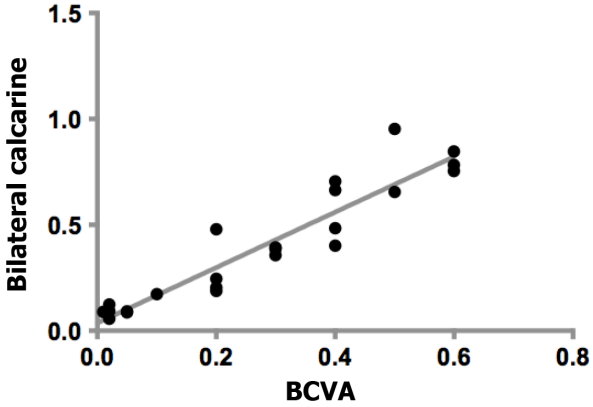

Figure 5 Correlations between the best-corrected visual acuity values and signal values in the bilateral calcarine. The best-corrected visual acuity value of the eyes of the diabetic retinopathy group showed a positive correlation with the signal value of the bilateral calcarine ($r = 0.938$, $P = 0.001$). BCVA: Best-corrected visual acuity.

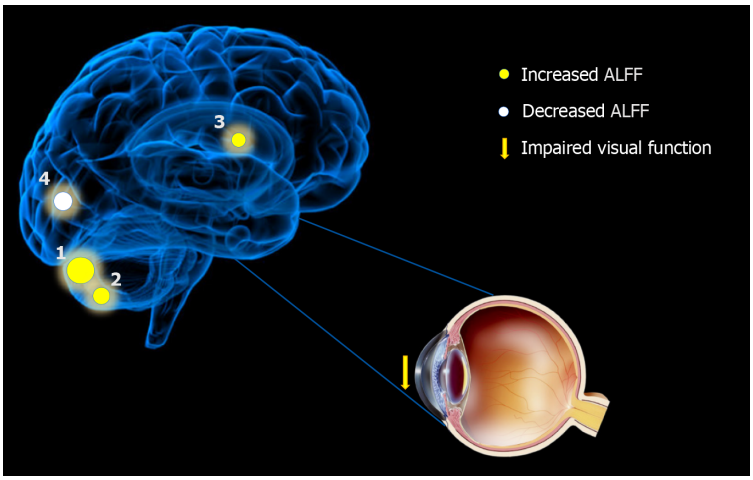

Figure 6 The amplitude of low-frequency fluctuation results of brain activity in the diabetic retinopathy group. Compared with the healthy controls, the amplitude of low-frequency fluctuation (ALFF) of the following regions in diabetic retinopathies were increased to various extents: 1-left cerebellum posterior lobe (t = 5.4338), 2-right cerebellum posterior lobe (t = 4.6875) and 3-right anterior cingulate (t = -4.4176), and decreased ALFF values in the 4-bilateral calcarine (t = -4.3494). The sizes of the spots denote the degree of quantitative changes. ALFF: Amplitude of low-frequency fluctuation.

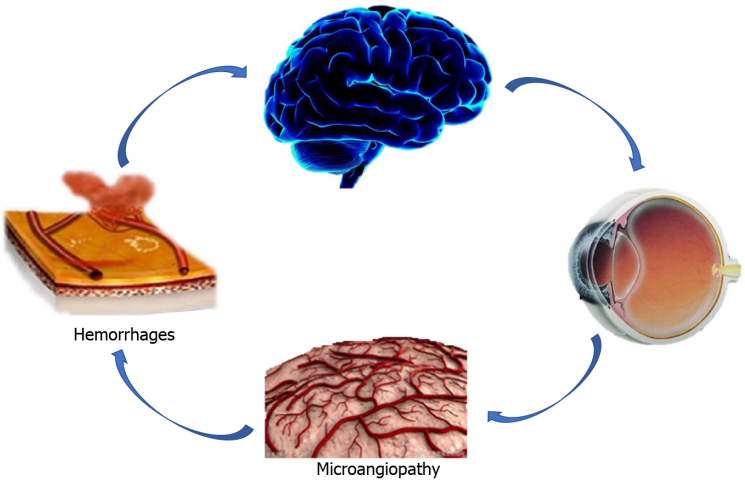

Figure 7 Relationship between magnetic resonance imaging images and clinical manifestations in diabetic retinopathy.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus; however, to date, there has been little analysis of the changes in brain function in patients with DR and their relationship to the clinical manifestations in the eye. This study is the first to examine brain changes in patients with DR using the amplitude of low-frequency fluctuation (ALFF).

Research motivation

The current diagnosis of DR mainly involves fundus fluorescein imaging for examination, and the direct connection between eyes, other manifestations, and the brain is still unknown. In this study, we employed the ALFF technique to investigate abnormal brain activity in DR patients and its relationship with clinical characteristics. Our research may help with understanding how DR disease develops.

Research objectives

We investigated the underlying ALFF of local characteristics of spontaneous brain activity in DR patients and their relationship with behavioral performance. Our findings suggested possible mechanisms of clinical manifestations and behavior in DR patients.

Research methods

Twenty-four DR patients and 24 healthy controls (HCs) matched for both age and sex were recruited. We measured and recorded the average ALFF values of DR patients and HCs and then classified them using receiver operating characteristic (ROC) curves.

Research results

We found that the ALFF values of both the left and right cerebellum posterior lobe and the right anterior cingulate gyrus were remarkably higher in the DR patients compared with the HCs, but DR patients also had lower values in the bilateral calcarine. ROC curve analysis of different brain regions demonstrated high accuracy of area under the curve analysis. However, there was no remarkable relationship between ALFF mean values for different regions and clinical presentations of DR patients.

Research conclusions

We hypothesized that DR may lead to alterations in visual cortical activity. Our results showed altered spontaneous activity in three regions of the brain in patients with DR. Abnormalities in low-frequency amplitudes in the brain may be associated with alterations in contralateral best-corrected visual acuity and depression in DR patients. These findings may suggest possible mechanisms of clinical manifestations and behaviors in DR patients.

Research perspectives

Our finding that DR may lead to multiple low-frequency amplitude frequency changes in the brain may facilitate our exploration of pathological mechanisms and disease progression in DR patients. However, the drawback is that the sample size was too small. Future studies should increase the sample size in order to ensure the validity of our findings.

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 February 15; 13(2): 110-125

DOI: 10.4239/wjd.v13.i2.110

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Large-scale functional connectivity predicts cognitive impairment related to type 2 diabetes mellitus

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Author contributions: Shi AP performed the data analysis, wrote the draft, conceived and designed the experiments, and rewrote some paragraphs in the introduction and discussion sections; Yu Y, Hu B, Li YT and Wang W obtained grants, conducted the experiments, and contributed to the writing and revision of the manuscript; Cui GB supervised the project, reviewed and edited the manuscript, and managed the submission process; all authors read, revised, and approved the final version of the manuscript.

Institutional review board statement: This research program was reviewed and approved by Ethics Committee of Tangdu Hospital.

Informed consent statement: Written informed consent was obtained from all participants before the experiment began.

Conflict-of-interest statement: We have no financial relationships to disclose.

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Abstract

BACKGROUND

Large-scale functional connectivity (LSFC) patterns in the brain have unique intrinsic characteristics. Abnormal LSFC patterns have been found in patients with dementia, as well as in those with mild cognitive impairment (MCI), and these patterns predicted their cognitive performance. It has been reported that patients with type 2 diabetes mellitus (T2DM) may develop MCI that could progress to dementia. We investigated whether we could adopt LSFC patterns as discriminative features to predict the cognitive function of patients with T2DM, using connectome-based predictive modeling (CPM) and a support vector machine.

AIM

To investigate the utility of LSFC for predicting cognitive impairment related to T2DM more accurately and reliably.

METHODS

Resting-state functional magnetic resonance images were derived from 42 patients with T2DM and 24 healthy controls. Cognitive function was assessed using the Montreal Cognitive Assessment (MoCA). Patients with T2DM were divided into two groups, according to the presence (T2DM-C; n = 16) or absence (T2DM-NC; n = 26) of MCI. Brain regions were marked using Harvard Oxford (HOA-112), automated anatomical labeling (AAL-116), and 264-region functional (Power-264) atlases. LSFC biomarkers for predicting MoCA scores were identified using a new CPM technique. Subsequently, we used a support vector machine based on LSFC patterns for among-group differentiation. The area under the receiver operating characteristic curve determined the appearance of the classification.

Data sharing statement: No additional data are available.

Supported by the National Natural Science Foundation of China, No. 81771815.

Country/Territory of origin: China

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: August 23, 2021
Peer-review started: August 23, 2021
First decision: December 4, 2021
Revised: December 10, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: February 15, 2022

P-Reviewer: Bansal A
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LYT

RESULTS

CPM could predict the MoCA scores in patients with T2DM (Pearson's correlation coefficient between predicted and actual MoCA scores, r = 0.32, P=0.0066 [HOA-112 atlas]; r = 0.32, P=0.0078 [AAL-116 atlas]; r = 0.42, P=0.0038 [Power-264 atlas]), indicating that LSFC patterns represent cognition-level measures in these patients. Positive (anti-correlated) LSFC networks based on the Power-264 atlas showed the best predictive performance; moreover, we observed new brain regions of interest associated with T2DM-related cognition. The area under the receiver operating characteristic curve values (T2DM-NC group *vs.* T2DM-C group) were 0.65-0.70, with LSFC matrices based on HOA-112 and Power-264 atlases having the highest value (0.70). Most discriminative and attractive LSFCs were related to the default mode network, limbic system, and basal ganglia.

CONCLUSION

LSFC provides neuroimaging-based information that may be useful in detecting MCI early and accurately in patients with T2DM.

Key Words: Connectome-based predictive modeling; Large-scale functional connectivity; Mild cognitive impairment; Resting-state functional magnetic resonance; Support vector machine; Type 2 diabetes mellitus

Core Tip: Large-scale functional connectivity (LSFC) patterns show unique characteristics. Abnormal LSFC patterns have been observed in patients with dementia or mild cognitive impairment. Patients with diabetes may develop mild cognitive impairment that could potentially progress to dementia. We assessed the applicability of LSFC-related discriminative features to predict the cognitive level of patients with type 2 diabetes mellitus using a connectome-based predictive modeling and support vector machine. We found that the application of these two techniques, based on LSFC patterns, to predict neurocognitive abilities, can complement conventional neurocognitive assessments and aid the management of type 2 diabetes mellitus.

Citation: Shi AP, Yu Y, Hu B, Li YT, Wang W, Cui GB. Large-scale functional connectivity predicts cognitive impairment related to type 2 diabetes mellitus. *World J Diabetes* 2022; 13(2): 110-125
URL: https://www.wjgnet.com/1948-9358/full/v13/i2/110.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i2.110

INTRODUCTION

Diabetes is a common and frequently occurring disease in clinical practice. It is a non-communicable disease that has gradually attracted increased attention worldwide, and its incidence is increasing with each passing year[1]. Mild cognitive impairment (MCI) occurs in nearly a quarter of patients with type 2 diabetes mellitus (T2DM) and is related to a significantly increased risk of developing dementia[2-5]. Patients with T2DM may present with deteriorated memory, attention, reagency, and execution[5]. In a cross-sectional study, Biessels *et al*[2] indicated that cognitive function was up to 0.3-0.5 SD lower than that of healthy controls (HC). T2DM leads to a variety of complications, as well as social health and economic problems[3]. In addition, 8.7% of patients with MCI rapidly progress to dementia each year[4]. In patients with T2DM who have a strong tendency to develop MCI and dementia, elucidating the neural mechanisms underlying cognitive dysfunction may assist in clinical identification and intervention, which can mitigate the progress of MCI. However, the mechanism underlying MCI in patients with T2DM warrants further exploration.

Previous studies using neuroimaging measures including the amplitude of low-frequency fluctuation[6], regional homogeneity[7], and functional connectivity[8,9] have reported potential neurobiological underpinnings in patients with T2DM and MCI. However, most of these studies focused on predetermined regions or networks,

including the default mode network (DMN), frontoparietal network (FPN), *etc*[9-12]. Additionally, most of these studies investigated group-wise differences among healthy participants, patients with T2DM with or without MCI, and patients with MCI alone, which limits the provided cognitive information.

Whole-brain functional connectivity, also known as large-scale functional connectivity (LSFC), presents vast functional interaction information between all pairs of brain nodes, which facilitates individual phenotypic prediction and the elucidation of individual differences in cognitive ability[13,14]. There are robust and reliable patterns of LSFC within several brain networks. Therefore, analyzing LSFC patterns may help elucidate the neural mechanisms underlying MCI. Abnormal LSFC patterns were reported in patients with Alzheimer's disease or MCI[15,16]. Additionally, recent functional magnetic resonance imaging (fMRI) studies have used LSFC to successfully predict individual behavioral and cognitive phenotypes, including psychiatric disorders[17], attention ability[18,19], intelligence ability[13], and treatment outcomes [20]. Zeng *et al*[17] used LSFC to discriminate patients with major depressive disorder from matched HC through machine learning (ML) based on LSFC. Similarly, Li *et al* [21] used ML and LSFC to classify patients with schizophrenia and HC.

Similar to fingerprints, individual LSFC patterns are highly unique and reliable, and could be applicable to the recognition of individual characteristics and cognitive function[13,18]. Therefore, some LSFC patterns could be considered as potential biomarkers for evaluating or identifying T2DM-related MCI. However, few studies have used LSFC combined with ML for assessing T2DM[8]. Thus, this study aimed to predict T2DM-related MCI at an individual level using connectome-based predictive modeling (CPM) and a support vector machine (SVM) combined with LSFC.

MATERIALS AND METHODS

Participants

All participants' informed consent forms were signed before the experiment began. LSFC was examined using resting state (rs)-fMRI data obtained from 42 patients with T2DM and 24 HC at Tangdu Hospital, Xi'an, Shaanxi, China, between October 1, 2016, and December 30, 2018. All participants were native Chinese speakers. T2DM was diagnosed based on the fasting blood glucose test (FBG; ≥ 7.0 mmol/L) and oral glucose tolerance test (2 h blood glucose ≥ 11.1 mmol/L after the test)[22], with the diagnosis being confirmed by clinical endocrinologists. Additionally, we administered the Chinese version of the Montreal Cognitive Assessment (MoCA)[23] and Mini-Mental State Examination (MMSE)[24] to classify the cognitive levels of all participants during this experiment. Trained physicians checked for MCI in patients with T2DM, who were divided into the T2DM-C (MoCA score ≤ 23 or MMSE score < 27, n = 16) and T2DM-NC (MoCA score ≥ 26 or MMSE score ≥ 27, n = 26) groups. A MoCA score ≤ 23[25] or MMSE score < 27[24] is indicative of cognitive impairment, whereas a MoCA score ≥ 26[25] or MMSE score ≥ 27[24] is considered cognitively normal. The exclusion criteria were as follows: other types of diabetes (type 1 diabetes or gestational diabetes); a history of severe encephalopathy (injury, tumor, inflammation, hemiplegia, or infarction) or myocardial infarction; central nervous system dysfunction or medical diseases that considerably affect neurological function, including acquired immune deficiency syndrome; taking drugs within 3 mo, such as psychoactive and steroid drugs; alcohol or drug addiction; pregnancy; contraindications for MRI examination, including cardiac pacemakers, artificial heart valves, and claustrophobia; body mass index (BMI) >35 kg/m^2 (because obesity impairs cognition); and unfavorable image quality or lack of coordination (head movement: translation >3.0 mm or rotation in any direction >3°). Similar exclusion criteria were adopted for the HC group.

MoCA scores

The MoCA is a quick evaluation scale for screening MCI[23,25]. Compared to the MMSE, the MoCA is more suitable for the screening and monitoring of MCI and dementia[23,25]. In our study, we used the Chinese version of the MoCA Basic (MoCA-BC) to assess the cognition level in patients with T2DM. MoCA-BC is recognized as a reliable test in cognitive screening, especially for milder forms of cognitive impairment across the education of all levels, especially in older Chinese adults, which has higher acceptance and better reliability[26]. It has good standard correlation validity (Pearson correlation coefficient MoCA-BC *vs*. MMSE = 0.787) and credible internal consistency (Cronbach alpha = 0.807)[26]. MoCA is scored on a 30-

point scale and comprises 11 items assessing orientation, attention, calculation, recall, and language. A MoCA score ≤ 23 indicates cognitive impairment and one ≥ 26 is considered to indicate cognitively normal.

Image acquisition and preprocessing

MRI data were obtained using a GE Discovery MR750 3.0T scanner (GE Medical Systems) with a brand-new coil system and high scanning speed. Foam pads were used to minimize head movement and earplugs were used to silence the scanner noise. During the acquisition phase, the participants were asked to relax, including at rest, to close their eyes, not think about anything, not allow being disturbed by others, and not to sleep. We recorded the blood oxygen level-dependent signals of spontaneous fluctuations during wakeful rest to assess brain activity. Three-dimensional brain volume (3D-BRAVO) and blood oxygen level-dependent sequences were used to obtain structural (including high-resolution T1-weighted images) and functional images, respectively. For details regarding the scanning parameters, see the Supplementary Material.

Data processing was conducted using DPABI (http://www.rfmri.org/)[27] and SPM (http://www.fil.ion.ucl.ac.uk/spm/), as well as homemade codes in MATLAB 2018a (MathWorks, Inc., Natick, MA). For details regarding rs-fMRI data preprocessing, see the Supplementary Material.

Functional connectivity network construction

Figure 1A shows the procedure for constructing functional brain networks. Brain regions were marked using three templates; namely, the Harvard Oxford (HOA-112) atlas[28], Automated Anatomical Labeling (AAL-116) atlas[29], and 264-region functional (Power-264) atlas introduced by Power *et al*[30]. We used the Pearson correlation analysis to calculate the mean time series of any two brain regions. Fisher's r-to-z transformation was applied to convert correlation coefficients to z-values. For each participant, an N × N (HOA-112 atlas, n = 112; AAL-116 atlas, n = 116; Power-264 atlas, n = 264) symmetric matrix was obtained.

We defined network nodes using the HOA -112, AAL-116, and Power-264 atlases. As previously described[31], 112 nodes were used to divide the brain into eight functional networks and the 116 nodes into nine macroscale brain regions. The eight functional networks included the visual (VN), sensory-motor (SMN), dorsal attention (DAN), ventral attention (VAN), limbic system, FPN, DMN, and basal ganglion (BG) networks. The nine macroscale brain regions included the VN, SMN, DAN, VAN, limbic system, FPN, DMN, BG, and cerebellar networks. Additionally, 264 nodes were divided into 14 Large-scale regions[30]. These nodes belonged to the DMN, salience, Cingulo-opercular Task Control (COTC), Fronto-parietal Task Control (FPTC), DAN, VAN, VN, auditory, Sensory/somatomotor Hand (SSH), Sensory/somatomotor Mouth (SSM) subcortical, Memory retrieval, cerebellar and uncertain networks. Details regarding the three templates can be found in Supplementary Tables 1, 2 and 3, as well as Supplementary Figure 1. Additionally, we calcula-ted the group mean functional connectivity matrices (FCMs) based on the three atlases for all three groups. Pairwise connectivity among the network nodes was described as a two-dimensional matrix using the functional connectivity matrix (FCM). In Supplementary Figure 2, the various regions of high (redder) and low (bluer) synchronization levels represent the FCM patterns of both patient groups, which were complex and similar. However, no evident between-group differences were found in the highlighted areas. Generally, the patient groups had fewer but stronger connections than the HC group.

Feature selection and connectome-based predictive modeling

Machine learning-based classification and prediction can allow the identification of clinically feasible neuroimaging biomarkers for cognitive decline in T2DM patients. We used CPM and SVM to obtain neuroimaging-based information potentially facilitating the clinical diagnosis of T2DM-C. Both analytical methods established links between the LSFC and several behavioral measures to generate a predictive model of behavioral data obtained from LSFC. However, SVM used the participants' group labels (*i.e.*, T2DM-C, T2DM-NC, and HC) as behavioral data while CPM used the MoCA scores in patients with T2DM.

Figure 1B illustrates the key CPM steps. Step 1: For each participant, CPM inputs comprised a set of M × M FCMs based on three atlases and a set of behavioral measures (here, MoCA scores). In the set of M × M FCMs, the number of brain regions or nodes is denoted by M; moreover, the between-node connection strength is associated with the matrix elements. Step 2 (feature selection): The Pearson's

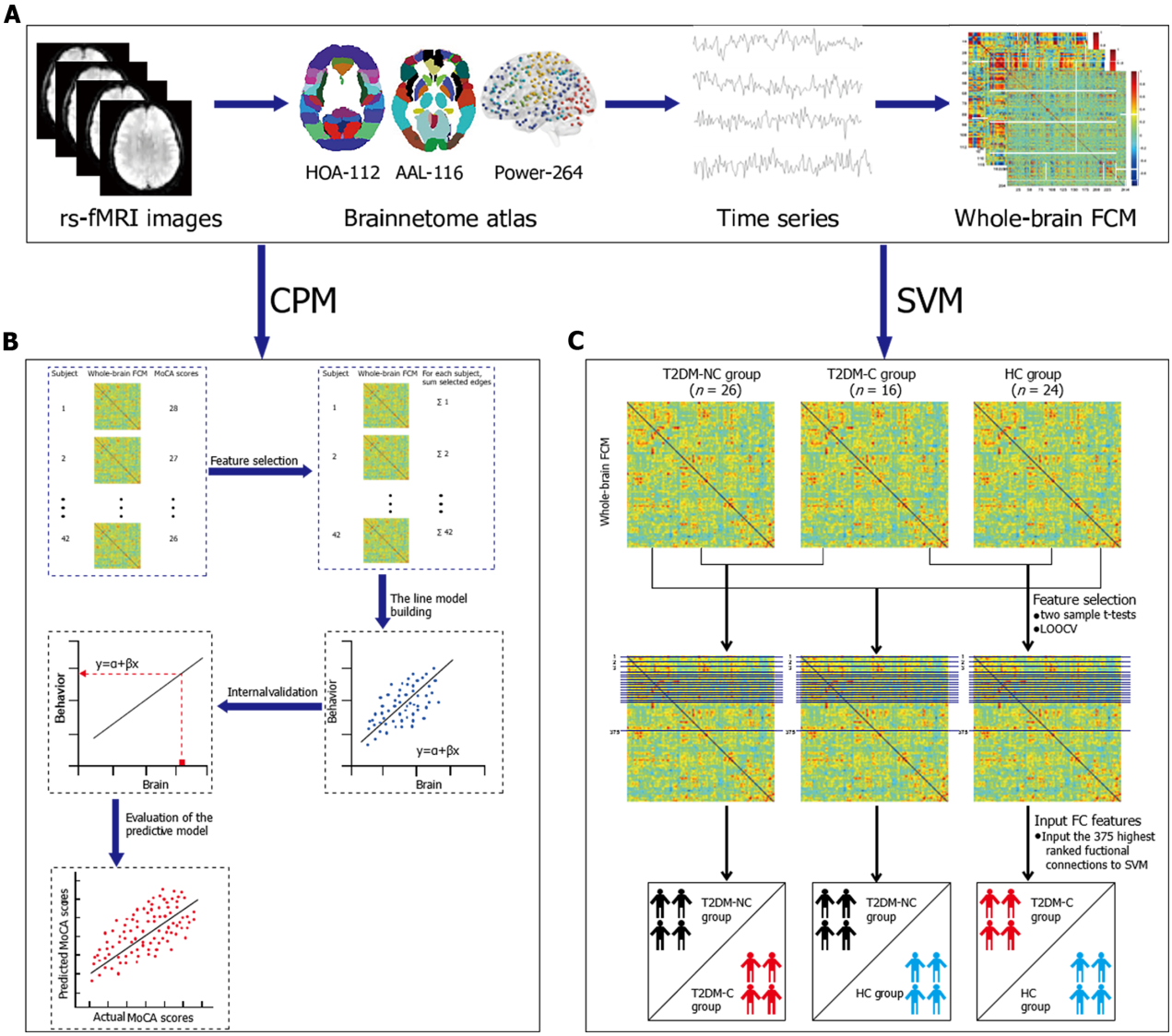

Figure 1 The prediction and classification flowchart. A: Relevant information from image preprocessing to feature identification; B: Detailed steps of connectome-based predictive modeling; C: Detailed steps of support vector machine. rs-fMRI: Resting state functional magnetic resonance imaging; HOA -112: Harvard Oxford atlas; AAL-116: Automated Anatomical Labeling; Power-264: 264-region functional atlas introduced by Power *et al*; FCM: Functional connectivity matrix; MoCA: Montreal Cognitive Assessment; T2DM: Type 2 diabetes mellitus; T2DM-C/T2DM-NC: Patients with T2DM with the presence/absence of mild cognitive impairment; HC: Healthy controls; LOOCV: Leave-one-out cross validation; FC: Functional connectivity.

correlation of each edge in the FCMs with the MoCA scores was computed. The most significant edges were pitched on by linear regression and subsequently merged into a single value for each participant. Based on the sign of the resultant r values with respect to a threshold of $P < 0.01$, they were separated into positive and negative tails (*i.e.*, positive and negative correlations, respectively, between the edge strength and MoCA scores)[18,32]. Subsequently, the positive and negative network strengths were computed by summing the edge strengths (*i.e.*, Z scores) for all edges in the positive and negative tails, respectively. Finally, we assessed the correlations of the positive and negative network strengths with the MoCA scores. Step 3 (line model building): Next, once the assumption of a linear relationship between the summary value of the connectivity data (independent variable) and the behavioral variable (dependent variable) was true, the predictive model was built; this was done separately for the positive and negative edge sets. Step 4 (prediction of novel participants): For each participant, the positive and negative edge sets were predicted by the behavioral measures. Given the limited sample size, leave-one-out cross validation (LOOCV) was applied separately to training and test data. The training and test datasets comprised N-1 and one participant, respectively. Step 5 (evaluation of predictive model): The comparison between the predicted and observed values can effectively evaluate the predictive model. Predictive accuracy was assessed using Pearson correlation analysis of the predicted and actual scores (r predicted-actual). Prediction performance was

assessed using permutation tests.

Feature selection and support vector machine

An SVM model was used to identify LSFC biomarkers for differentiating between the T2DM-NC/T2DM-C, T2DM-NC/HC, and T2DM-C/HC groups. SVM is the most used classification algorithm in ML[33]. For instance, we trained an SVM model using the training dataset to map the set of features of respective labels when given a specific feature (*e.g.*, LSFC) and label (*e.g.*, T2DM and HC). Therefore, given a new dataset, the SVM can be used to predict its label (group). The performance of these models was estimated through LOOCV using measures of accuracy, sensitivity, the receiver operating characteristic (ROC) curve, and the area under the ROC curve (AUC). The use of SVM was dependent on the Statistics and Machine Learning Toolbox in MATLAB 2018a. Figure 1C illustrates the detailed steps of SVM. Step 1: For further selection, some lower triangle elements of each FCM were extracted. The feature space spanned $(112 \times 111)/2 = 6216$, $(116 \times 115)/2 = 6670$, and $(264 \times 263)/2 = 34716$ dimensional functional connections for the HOA-112 atlas, AAL-116 atlas, and Power-264 atlas, respectively. Step 2 (feature selection): As reported previously[17,21], the analysis mentioned above was performed *via* two-sample t-tests and LOOCV. Specifically, 66 observations (FCMs with among-group differences) were subdivided into 66 folds. For each fold, the features were ranked in descending order based on the absolute between-group t values, followed by selection of the most discernible connections (from 1 to 375). Step 3 (input the classification features): We input the 375 highest-ranked functional connections into the SVM classifier model trained by LOOCV using the training data. Step 4 (evaluate the appearance of the SVM model using ROC curves and AUC): Sensitivity and specificity refer to the proportion of true positive and negative samples, which are associated with the diagnostic values.

Statistical analysis

SPSS (version 20.0; SPSS, Chicago, IL, USA) was used for statistical analysis. $P < 0.05$ indicated statistical significance. Grouped non-continuous data, including sex, were compared using chi-squared tests. We used one-way analysis of variance to evaluate normally distributed quantitative data, including education, HbA1c (%), BMI, self-rating anxiety scale (SAS) scores, and self-rating depression scale (SDS) scores. The SDS is a simple, 20-question scale that reflects depressive mood, physical symptoms, psychomotor behavior, and psychological symptom experience based on how one feels over the course of a week. Since it is self-administered, the test is widely used and does not require others' participation. The SAS is a self-rating scale containing 20 items (hoping to elicit 20 symptoms) divided into 4 grades. The main evaluation item is the frequency of the occurrence of the defined symptoms. The criteria are: "1" the symptoms occur a little or none of the time; "2" a small part of the time; "3" a lot of the time; "4" most or all the time. The SAS is intended for adults with symptoms of anxiety. At the same time, it has a wider applicability than the SDS. For values with significant among-group differences, the least significant difference was used to perform post hoc comparisons between each group pair. Non-normally distributed continuous quantitative data, including age, FBG, waist-to-hip ratio (WHR), systolic pressure, diastolic pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, urinary microalbumin, duration of diabetes, MMSE scores, and MoCA scores, are expressed as the median (minimum, maximum). Between-group and among-group differences in non-normally distributed data were evaluated using the Mann-Whitney U test and Kruskal-Wallis non-parametric comparisons, respectively. However, the Kruskal-Wallis test could not perform pairwise comparisons among the three groups, which were performed directly through SPSS version 20.0.

P-values were corrected, and multiple comparison issues were addressed by permutation tests[34] performed by randomly assigning participants to two groups 5,000 times. When regional volume and eigenvector centrality values did not belong to the 95% of the null distribution of permutation tests ($P < 0.05$, corrected), the differences were considered significant. All the analyses mentioned above were performed using MATLAB.

RESULTS

Demographic and clinical characteristics

Table 1 summarizes the clinical and demographic characteristics of the T2DM-NC, T2DM-C, and HC groups. No significant between-group differences were found in age, sex distribution, BMI, blood pressure, total cholesterol, triglycerides, urinary microalbumin, duration of diabetes, SAS scores, and SDS scores. Compared with the T2DM-C group, the T2DM-NC and HC groups had higher levels of education (P = 0.040 and P = 0.015, respectively), MMSE scores (P < 0.001 and P = 0.002, respectively), and MoCA scores (P < 0.001 and P = 0.001, respectively). No significant differences were found in education, MMSE scores, and MoCA scores between the T2DM-NC and HC groups. Furthermore, compared with the HC group, the T2DM-C/NC groups had higher levels of HbA1c (P < 0.001), HDL cholesterol (P = 0.005, P = 0.006), FBG (P = 0.001, P < 0.001), and WHR (P = 0.017, P = 0.002, respectively). No significant differences were found in the levels of HbA1c, HDL cholesterol, FBG, and WHR between the T2DM-C/NC groups.

Individualized prediction of T2DM outcome

Based on the fMRI data, we found that the CPM, which was based on positive network strength, could significantly predict the participants' MoCA scores (Pearson's correlation of predicted and observed MoCA scores, r = 0.32, P = 0.0066 [HOA-112 atlas]; r = 0.32, P = 0.0078 [AAL-116 atlas]; r = 0.42, P = 0.0038 [Power-264 atlas]; see Table 2 and Figure 2). However, the predictions were not significant in the negative network model. Compared to the random label (P < 0.01), permutation tests (repetition times: 5000) indicated the higher actual classification accuracy.

For the HOA-112 atlas, between-network connectivity in the VAN, DMN, and SMN was crucially involved in predicting the MoCA scores in patients with T2DM. For the AAL-116 atlas, significantly discriminative LSFCs were mainly located across the limbic system, DMN, VN, BG, and cerebellum. For the Power-264 atlas, the most significantly predictive LSFCs were those between the VN and SSH. Overall, highly discriminative LSFCs were mainly located in the DMN, limbic system, BG, and VN.

Network anatomy predicts MoCA scores

Next, we investigated the neuroanatomy of positive MoCA networks. Figure 3A-C show a circle plot visualization for edges, which comprises the positive MoCA networks. These figures present the general neurocognitive composition of positive MoCA networks, which are indicative of the advanced descriptions of the brain regions involved. Figure 3D-F show glass brain plots displaying the above LSFCs localized in the 3D brain space. These figures indicate that these LSFCs, which were used to predict the differences between MoCA scores, were not located in specific brain regions but distributed throughout the brain.

Individualized classification of T2DM outcomes

Table 3 and Figure 4 show the ROC curves and AUC values. We selected 375 functional connections using the LOOCV after achieving the highest performance. Although the SVM model did not achieve good performance in three two-category classifications, the highest performance was achieved in discriminating between the T2DM-C/NC groups using the 375 highest-ranked functional connections (HOA-112 atlas: AUC=0.70, specificity = 0.69, sensitivity = 0.73, P = 0.0144; AAL-116 atlas: AUC = 0.65, specificity = 0.69, sensitivity = 0.65, P = 0.0556; Power -264 atlas: AUC = 0.70, specificity = 0.63, sensitivity = 0.77, P = 0.0160).

For the HOA-112 atlas, between-network connectivity in the BG, SMN, and FPN was crucially involved in discriminating between the T2DM-C/NC groups. For the AAL-116 atlas, the most discriminative and attractive LSFCs were located between the limbic system and the BG, as well as between the DMN and cerebellum. For the Power-264 atlas, the most significantly predictive functional connections were between the DMN and FPTC network. Overall, the DMN and BG were crucially involved in differentiating between the T2DM-C/NC groups.

Network anatomy in the classification of T2DM-C and T2DM-NC

Next, we visualized the neuroanatomical location of the network identified by classification (T2DM-C group *vs*. T2DM-NC group). Figure 5A-C demonstrate the network identified by classification after grouping the edges into macroscale brain regions. Figures 5D-F show glass brain plots displaying the same LSFCs localized in the 3D brain space; these figures indicate that these LSFCs, which were also used to predict

Table 1 Demographic and clinical characteristics of these three groups

Characteristics	T2DM-NC (*n* = 26)	T2DM-C (*n* = 16)	HC (*n* = 24)	*P* value
Age (yr)[2]	51 (34, 65)	54 (39, 67)	49 (26, 59)	0.227
Female/Male	4/22	6/10	9/15	0.153
Education (yr) [1]	12.88 ± 2.55	10.81 ± 2.76	13.38 ± 3.88	0.040
HbA1c (%)[1]	8.13 ± 1.87	9.06 ± 1.77	5.66 ± 0.33	0.000
FBG (mg/dL) [2]	7.85 (4.20, 15.80)	7.60 (3.60, 11.70)	5.20 (4.80, 6.80)	0.000
BMI (kg/m2) [1]	25.26 ± 2.43	24.90 ± 2.97	23.80 ± 2.41	0.779
WHR[2]	0.91 (0.76, 0.96)	0.91 (0.86, 0.96)	0.87 (0.78, 0.93)	0.004
Blood pressure (mmHg)				
SP[2]	128.00 (105.00, 150.00)	120.00 (101.00, 150.00)	128.00 (100.00, 181.00)	0.836
DP[2]	80.00 (60.00, 90.00)	80.00 (60.00, 90.00)	80.00 (67.00, 118.00)	0.432
Total cholesterol[2]	4.04 (2.76, 6.69)	4.21 (2.63, 5.71)	4.43 (3.69, 5.39)	0.407
HDL cholesterol[2]	1.35 (0.43, 6.60)	1.26 (0.53, 8.08)	0.94 (0.71, 1.64)	0.001
Triglycerides (mg/dL)[2]	1.75 (0.43, 6.60)	1.26 (0.53, 8.08)	2.06 (0.87, 6.41)	0.457
UMA (μg/min)[2]	12.45 (1.00, 342.70)	15.95 (7.00, 299.00)	13.65 (0.40, 58.60)	0.706
Duration of diabetes (mo)[2]	96.00 (0.25, 180.00)	24.00 (0.25, 228.00)		0.515
MMSE[2]	29.00 (27.00, 30.00)	26.00 (23.00, 29.00)	28.00 (27.00, 30.00)	0.000
MoCA[2]	27.00 (25.00, 30.00)	24.00 (18.00, 30.00)	27.00 (24.00, 30.00)	0.000
SAS[1]	41.62 ± 7.12	43.75 ± 7.26	39.54 ± 7.00	0.190
SDS[1]	46.12 ± 6.87	45.31 ± 8.46	41.71 ± 10.07	0.172

[1]Data are presented as mean ± SD.
[2]Data are presented as median (minimum, maximum).
P < 0.05 was considered significant. T2DM: Type 2 diabetes mellitus; T2DM-C/T2DM-NC: Patients with T2DM with the presence/absence of mild cognitive impairment; HC: Healthy controls; HbA1c: Glycosylated hemoglobin A1c; FBG: Fasting blood glucose; BMI: Body mass index; WHR: Waist-to-Hip Ratio; SP: Systolic pressure; DP: Diastolic pressure; HDL: High density lipoprotein; UMA: Urinary microalbumin; MMSE: Mini-mental state examination; MoCA: Montreal cognitive assessment; SAS: Self-rating anxiety scale; SDS: Self-rating depression scale.

Table 2 Prediction outcome

Brain atlas	Correlation coefficient	*P* value
HOA-112 atlas	0.32	0.0066
AAL-116 atlas	0.32	0.0078
Power-264 atlas	0.42	0.0038

HOA-112: Harvard Oxford atlas; AAL-116: Automated Anatomical Labeling; Power-264: 264-region functional atlas introduced by Power *et al*.

the differences between MoCA scores, were not located in specific brain regions but distributed throughout the brain.

DISCUSSION

The present study examined whether we could adopt LSFC patterns as discriminative features to classify and predict cognitive impairment related to T2DM with a high degree of accuracy. Compared to neuropsychological scales, which may be unreliable and subjective, it is evident from our results that LSFC is useful in the early detection of MCI related to T2DM. Our results indicate that functional networks contain clinically relevant cognition-related information, which is defined in a data-driven

Table 3 Classification outcome

Group	Brain atlas	AUC	Specificity	Sensitivity	*P* value
T2DM-NC *vs* T2DM-C	HOA-112 atlas	0.70	0.69	0.73	0.0144
	AAL-116 atlas	0.65	0.69	0.65	0.0556
	Power-264 atlas	0.70	0.63	0.77	0.0160
T2DM-NC *vs* HC	HOA-112 atlas	0.54	0.75	0.42	0.3122
	AAL-116 atlas	0.53	0.58	0.54	0.3804
	Power-264 atlas	0.56	0.58	0.58	0.2478
T2DM-C *vs* HC	HOA-112 atlas	0.54	0.63	0.56	0.3152
	AAL-116 atlas	0.72	0.67	0.75	0.0096
	Power-264 atlas	0.70	0.79	0.63	0.0184
T2DM *vs* HC	HOA-112 atlas	0.67	0.63	0.69	0.0144
	AAL-116 atlas	0.63	0.58	0.64	0.0444
	Power-264 atlas	0.50	0.50	0.67	0.4898

T2DM: Type 2 diabetes mellitus; T2DM-C/T2DM-NC: Patients with T2DM with the presence/absence of mild cognitive impairment; HC: Healthy controls; AUC: The area under the receiver operating characteristic curve; HOA-112: Harvard Oxford atlas; AAL-116: Automated Anatomical Labeling; Power-264: 264-region functional atlas introduced by Power *et al*.

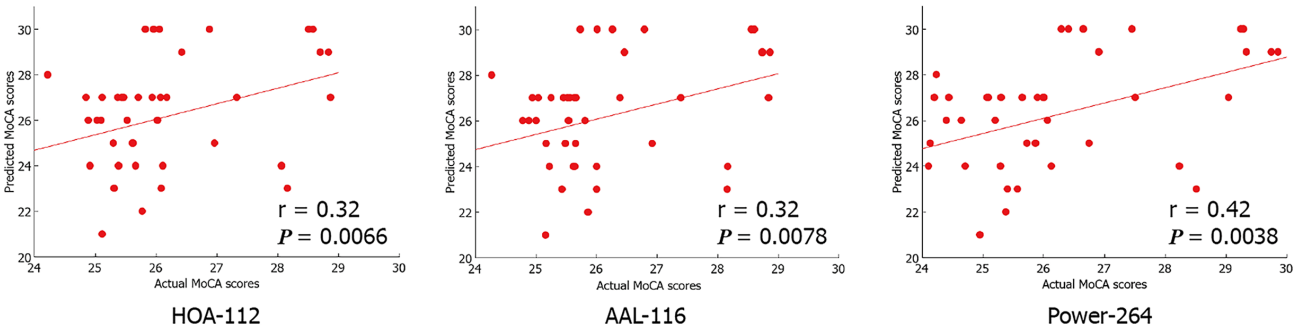

Figure 2 The connectome-based predictive modeling predicted the Montreal Cognitive Assessment scores. Scatterplot of predicted the Montreal Cognitive Assessment (MoCA) scores vs. actual MoCA scores. Predicted scores were derived from edges positively correlated with prediction (positive network). *r*: The *r* value of Pearson's correlation of predicted the MoCA scores and actual MoCA scores; *P*: *P* values from permutation tests (5000 times); T2DM: Type 2 diabetes mellitus; T2DM-C/ T2DM-NC: Patients with T2DM with the presence/absence of mild cognitive impairment; HOA -112: Harvard Oxford atlas; AAL-116: Automated Anatomical Labeling; Power-264: 264-region functional atlas introduced by Power *et al*.

manner and has the potential to be a biomarker to assess the degree of cognitive decline related to T2DM.

T2DM is often associated with cognitive impairment and a higher dementia risk. Patients with T2DM may present with deteriorated memory, attention, reagency, and execution[2-5]. However, the exact pathophysiological mechanisms underlying T2DM-related cognitive dysfunction remain unclear, which impedes the development of preventive treatments. We analyzed resting-state fMRI data using the CPM and SVM. We computed the LSFC patterns using three types of functional brain atlases that separately comprised 112, 116, and 264 nodes covering the whole brain. The SVM-based classification results were not as expected; the exact reasons for which remain unclear. However, the CPM-based prediction results were positive, with exciting prospects. There have been no previous CPM studies on patients with T2DM; moreover, this is the first study to identify LSFC as an imaging biomarker for predicting T2DM-related MCI using CPM. CPM can reliably predict the participants' MoCA scores, which was based on positive network strength (r = 0.32, P = 0.0066 [HOA-112 atlas]; r = 0.32, P = 0.0078 [AAL-116 atlas]; r = 0.42, P = 0.0038 [Power-264 atlas]). Highly discriminative and attractive LSFCs were mainly located within the DMN, limbic system, BG, VN, or across these regions. Our findings suggest that the resting-state LSFC can reveal T2DM-related MCI, which could be more reliable than

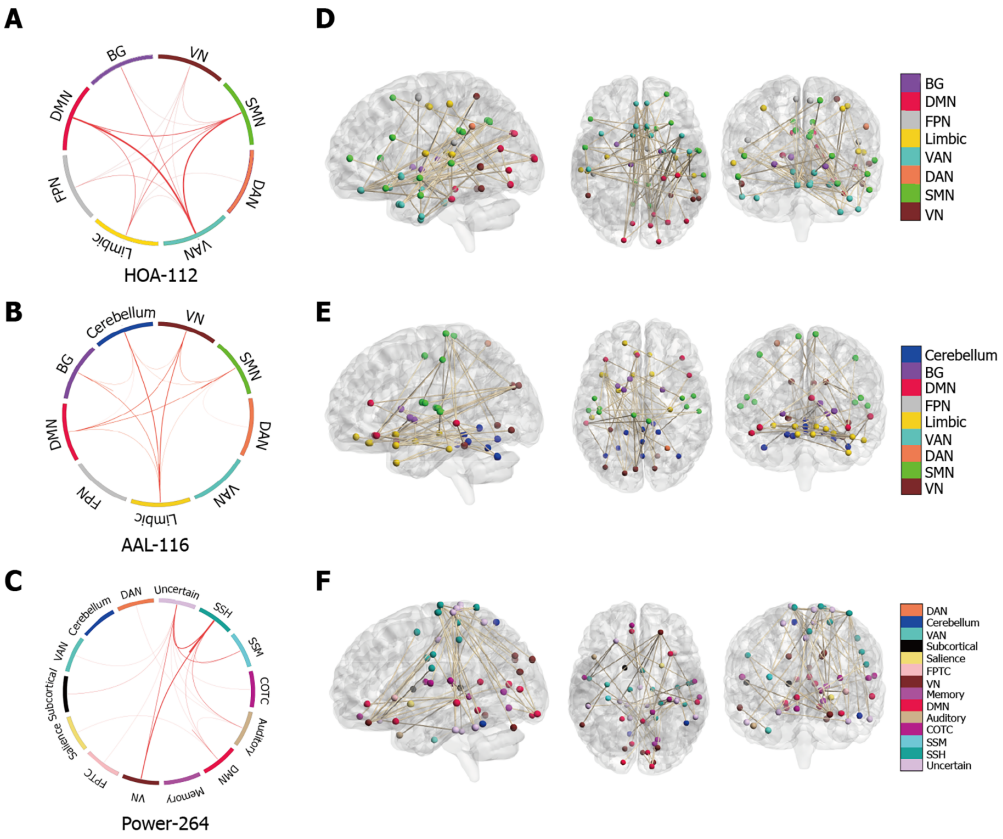

Figure 3 Functional connections predicting individual Montreal Cognitive Assessment scores based on three atlases. A-C: On the far left of the image above, edges were classified as macroscale brain regions, and visualized by circle plots, in which nodes are grouped based on their anatomic location. The resting-state network (RSN) of the brain based on three templates is represented by a rectangle on the circumference of the big circle. The lines connecting two rectangles represent the connections between the corresponding one or two RSNs, including inter-network connections and intra-network connections. The thickness of the line represents the weight (*i.e.,* connectome-based predictive modeling weight) of the connection. The thicker the line, the larger the weight. This visualization was created using Circos (http://circos.ca/). D-F: On the right of the image above, the same edges are visualized in the brain. The lines represent edges connecting the spheres, which in turn represent nodes. A legend indicating the approximate anatomic 'lobe' is shown in the far right side of the figure. HOA -112: Harvard Oxford atlas; AAL-116: Automated Anatomical Labeling; Power-264: 264-region functional atlas introduced by Power *et al*; VN: Visual Network; SMN: Sensory-motor Network; DAN: Dorsal Attention Network; VAN: Ventral Attention Network; Limbic: Limbic System; FPN: Fronto-parietal network; DMN: Default mode network; BG: Basal ganglia; SSH: Sensory somatomotor hand; SSM: Sensory somatomotor mouth; COTC: Cingulo-opercular task control; Memory: Memory retrieval; FPTC: Fronto-parietal task control.

standardized neuropsychological scales. There is significant interest in using the LSFC to predict human behavior. We found that the LSFC-based CPM could effectively predict the MoCA scores in patients with T2DM. The prediction of neurocognitive abilities from CPM can complete the conventional assessments. The CPM-related positive network was used as a T2DM-related MCI connectivity measure and showed favorable results based on the Pearson correlation coefficient. CPM can predict individua behaviors or characteristics by LSFC, which is novel and data-driven[13,35]; moreover, it can successfully predict the number of psychiatric and psychological phenotypes[32,36]. CPM can isolate brain "fingerprints" that identify individual participants from a group[13], as well as predict personality traits[32], sustained attention[18,37], treatment outcomes[20], and cognitive dysfunction[8,38]. However, unlike previous studies on fluid intelligence[13] and attention[18], where the positive and negative networks showed comparable predictive performance, we found that the negative network showed an unfavorable predictive performance.

Regarding the functional anatomy of the edges, which is most relevant to individual differences in the degree of cognition, we paid more attention to the CPM-positive network. Moreover, lower MoCA scores were associated with higher network strength, indicating more severe cognitive dysfunction. This suggests that the cognitive decline in T2DM patients may involve abnormal connectivity among these different resting-state networks. For both prediction and classification, most significantly discriminative functional connections were related to the DMN, limbic system, and the BG.

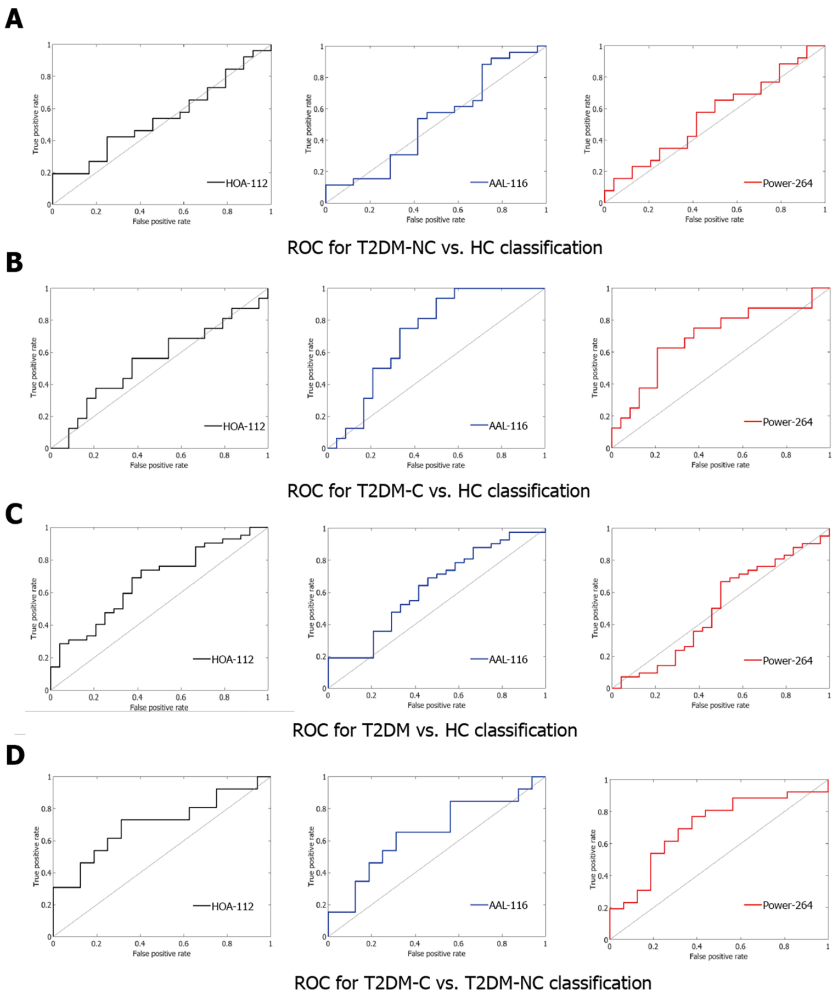

Figure 4 Classification efficiency of support vector machine based on three atlases. The classification effect was not very ideal. A: The area under curve (AUC) value of patients with type 2 diabetes mellitus (T2DM) with the absence of mild cognitive impairment (T2DM-NC) Verus healthy controls (HC) group was 0.54 [Harvard Oxford (HOA-112) atlas], 0.53 [Automated Anatomical Labeling (AAL-116) atlas], 0.56 [264-region functional (Power-264) atlas]; B: the AUC value of patients with T2DM with the presence of mild cognitive impairment (T2DM-C) Verus HC group was 0.54 (HOA-112 atlas), 0.72 (AAL-116 atlas), 0.70 (Power-264 atlas); C: the AUC value of T2DM Verus HC group was 0.67 (HOA-112 atlas), 0.63 (AAL-116 atlas), 0.50 (Power-264 atlas); D: the AUC value of T2DM-C Verus T2DM-NC group was 0.70 (HOA-112 atlas and Power-264 atlas), 0.65(AAL-116 atlas). ROC: receiver operating characteristic curve.

The DMN is activated during wakeful rest and deactivated during cognitive task execution; further, it is involved in cognitive processing[8,11]. The DMN comprises several brain regions, including the anterior cingulate cortex; medial prefrontal cortex; and the medial, lateral, and inferior parietal cortices[39], which are involved in constructing self-related mental simulations, including recalling the past, thinking about the future, and understanding others' perspectives[8,11]. Cognitive impairment in T2DM is related to reduced connectivity in cognition-related networks, most prominently in the DMN[40]. Changes in brain structure and function are associated with the deterioration of cognition; moreover, blood glucose fluctuations (hyperglycemia or hypoglycemia) may be related to T2DM-related brain changes[41]. Repeated hyperglycemia and hypoglycemia can lead to a variety of metabolic and molecular changes that eventually lead to widespread changes in brain cells. However, the exact causes of T2DM-related changes in the DMN are unclear. Specific alterations in functional connectivity may contribute to cognitive decline in patients with T2DM and may represent a promising biomarker.

The cingulate/paracingulate gyrus and parahippocampal gyrus are indispensable to the functioning of the limbic system. They are crucially involved in learning, emotion, memory, and other processes. A recent meta-analysis, including 15 structural studies and 16 functional studies, reported decreased global and regional gray matter volume in the limbic system of patients with T2DM, which could be associated with poor cognitive performance[42]. The results from some studies indicate that the changes in limbic regions, especially in dendritic structures, inhibit the formation of the spinal cord due to the chronic hyperglycemia; moreover, they may also disrupt the

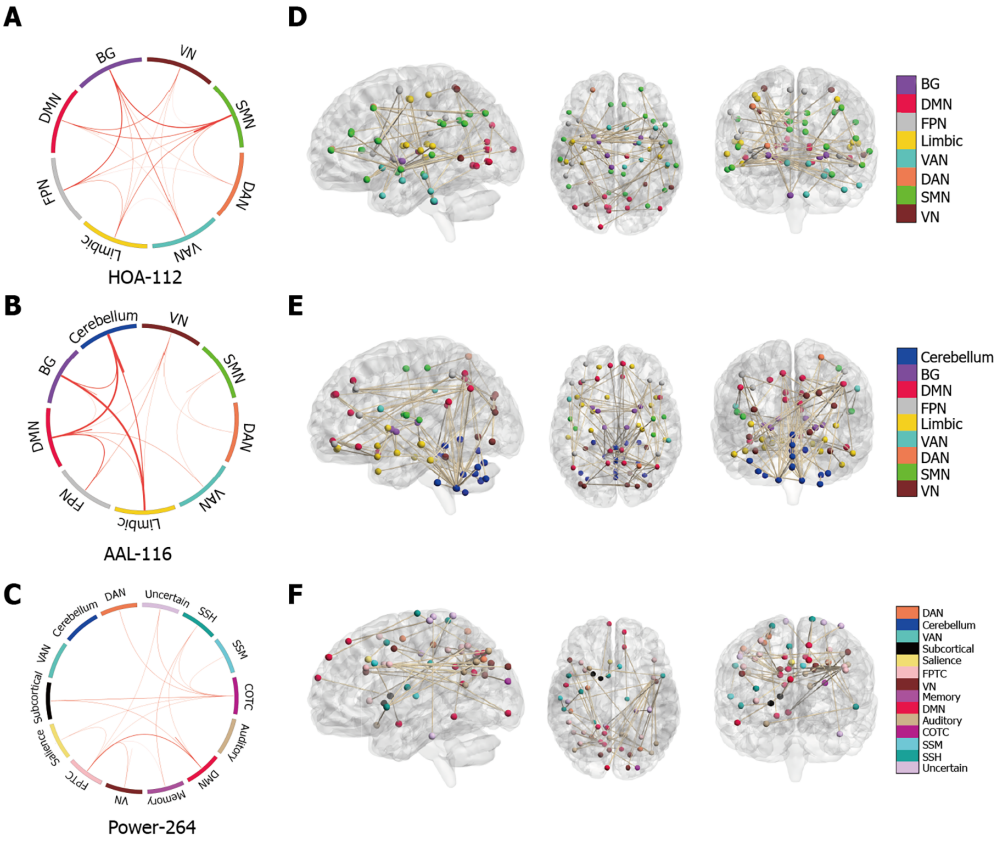

Figure 5 Functional connections classifying patients with type 2 diabetes mellitus with the present of mild cognitive impairment and patients with type 2 diabetes mellitus with the absence of mild cognitive impairment based on three different atlas. A-C: On the far left of the image above, edges were classified as macroscale brain regions and were visualized using circle plots, in which nodes are grouped based on anatomic location. The resting-state network (RSN) of brain based on three atlas is represented by a rectangle on the circumference of the big circle. The lines connecting two rectangles represent the connections between the corresponding one or two RSN, including inter-network connections and intra-network connections. The thickness of the line represents the weight (*i.e.*, support vector classification weight) of the connection. The thicker the line, the larger the weight. This visualization was created using Circos (http://circos.ca/); D-F: On the right of the image above, the same edges are visualized on brains. The lines represent edges connecting the spheres, which represent nodes. A legend indicating the approximate anatomic 'lobe' is shown in the far right side of picture. VN: Visual network; SMN: Sensory-motor network; DAN: Dorsal attention network; VAN: Ventral attention network; Limbic: Limbic system; FPN: Fronto-parietal network; DMN: Default mode network; BG: Basal ganglia; SSH: Sensory Somatomotor hand; SSM: Sensory somatomotor mouth; COTC: Cingulo-opercular task control; Memory: Memory retrieval; FPTC: Fronto-parietal task control.

processes of memory and learning[43,44]. In addition, multi-timescale variability of abnormal glucose regulation may be associated with poor cognitive function in patients with T2DM, which may be attributed to the gray matter atrophy in the limbic region[45].

Different structures within the basal ganglia, which is involved in movement regulation, play different roles in various diseases. Lesions in the basal ganglia region mainly result in abnormal movement (increased or decreased movement) and changes in muscle tone (increased or decreased). The basal ganglia represent an important neural functional area, closely related to sensory, motor, visual, behavioral and other functions. This area has a high incidence of stroke. Parkinson's disease and Huntington's disease are among the most studied diseases in the area[46]. There is no adequate evidence regarding a relationship between the basal ganglia and T2DM; however, patients with T2DM-C have been shown to have severely impaired overall network efficiency, with decreased lymph node efficiency and connections in multiple regions, including the limbic system and BG[47]. Additionally, a meta-analysis reported reduced overall brain volume and BG atrophy in patients with T2DM[48]. Basal ganglia changes in diabetics typically occur in hyperglycemic osmotic states in older Asian women[49]. Attributable causes of dyskinesia in diabetic patients include hyperglycemia, high viscosity, changes in brain gamma aminobutyric acid metabolism, diabetic angiopathy, and cytotoxic edema. High blood sugar and viscosity can break down the blood-brain barrier, leading to ischemia. Taken together, T2DM-related cognitive impairment may involve abnormal connection patterns across the DMN, limbic system, and BG.

Overall, our study has three main features. First, this is the first study to successfully apply CPM in patients with T2DM for identifying neuroimaging biomarkers associated with cognitive impairment. Second, unlike most previous studies that performed between-group comparisons using *a priori* defined brain regions/networks[8,9,11,40], we performed whole-brain bottom-up analyses. Therefore, our method could facilitate the identification of crucial features for predicting cognitive performance at an individual level. Third, we used three brain templates, namely the HOA-112, AAL-116, and Power-264 atlases, to demonstrate the predictive utility of CPM for determining T2DM-related cognitive impairment from different perspectives. Still, there are some limitations in this study. First, doubling our sample size might have increase the generalizability of our results. Second, we only used rs-fMRI data, whereas other modalities like structural MRI and diffusion-weighted imaging might provide complementary information to improve the quantification of brain networks. Finally, according to our findings, the neurobiological changes of T2DM can be reflected by the resting-state brain network. More in-depth and longitudinal studies are required to elucidate the specific influence on T2DM pathogenesis, especially T2DM-related problems in thought processing.

CONCLUSION

This study used the CPM method to identify LSFC patterns, including connections across the DMN, limbic system, and BG, as potential biomarkers for overall cognitive status in patients with T2DM. LSFC provided neuroimaging-based information that could clinically predict the MoCA scores in patients with T2DM. Applying CPM based on LSFC for predicting neurocognitive abilities can complement conventional neurocognitive assessments and facilitate the management of patients with T2DM.

ARTICLE HIGHLIGHTS

Research background

Whole-brain functional connectivity patterns, or large-scale functional connectivity (LSFC) patterns, are both highly unique and reliable in each individual, and similar to a fingerprint, can identify individual differences in personality traits or cognitive functions. Abnormal LSFC patterns have been found in patients with dementia, as well as in those with mild cognitive impairment (MCI), which predicted their cognitive performance. It has been reported that patients with type 2 diabetes mellitus (T2DM) may develop MCI that could progress to dementia. We assessed the applicability of LSFC-related discriminative features to predict the cognitive level of patients with T2DM using a connectome-based predictive modeling (CPM) and support vector machine (SVM).

Research motivation

Whether machine learning techniques like CPM and SVM could utilize LSFC patterns to predict T2DM-related MCI with a high degree of accuracy remains unclear.

Research objectives

To investigate the utility of LSFC for more accurately and reliably predicting the cognitive impairment related to T2DM.

Research methods

Resting-state functional magnetic resonance images were derived from 42 patients with T2DM and 24 healthy controls. Cognitive function was assessed using the Montreal Cognitive Assessment (MoCA). Patients with T2DM were divided into two groups, according to the presence (T2DM-C; n = 16) or absence (T2DM-NC; n = 26) of MCI. Brain regions were marked using the Harvard Oxford (HOA-112), automated anatomical labeling (AAL-116), and 264-region functional (Power-264) atlases. LSFC biomarkers for predicting MoCA scores were identified using a new CPM technique. Subsequently, we used the SVM based on LSFC patterns for among-group differentiation. The area under the receiver operating characteristic curve determined the classification appearance.

Research results

CPM could predict MoCA scores in patients with T2DM, indicating that LSFC patterns represent cognition-level measures in these patients. Positive (anti-correlated) LSFC networks based on the Power-264 atlas showed the best predictive performance (r=0.42, *P*=0.0038); moreover, we observed new brain regions of interest associated with T2DM-related cognition. The area under the receiver operating characteristic curve values (T2DM-NC group *vs*. T2DM-C group) were 0.65-0.70, with LSFC matrices based on HOA-112 and Power-264 atlases having the highest value (0.70). Most discriminative and attractive LSFCs were related to the default mode network, limbic system, and basal ganglia.

Research conclusions

LSFC provides neuroimaging-based information that may be useful in detecting MCI early and accurately in patients with T2DM and therefore assist with T2DM management.

Research perspectives

Our study provides promising evidence that LSFC can reveal cognitive impairment in patients with T2DM, although further development is needed for clinical application.

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World Journal of Diabetes

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World J Diabetes 2022 February 15; 13(2): 126-128

DOI: 10.4239/wjd.v13.i2.126

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Inflammatory bowel disease and diabetes: Is there a link between them?

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Author contributions: Sang MM wrote the letter; Sun ZL and Wu TZ revised the letter.

Conflict-of-interest statement: The authors declare that they have no conflict-of-interest.

Country/Territory of origin: Australia

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

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Abstract

Patients with inflammatory bowel disease (IBD) are reported to have an increased risk of diabetes. IBD therapies may also modulate blood glucose substantially. These observations are indicative of mechanistic connection(s) between IBD and diabetes.

Key Words: Inflammatory bowel disease; Abnormal glucose metabolism

Core Tip: Inflammatory bowel disease is associated with an increased risk of diabetes. Mechanistic insights into their common pathogenesis may render novel therapeutic targets for these major chronic disorders.

Citation: Sang MM, Sun ZL, Wu TZ. Inflammatory bowel disease and diabetes: Is there a link between them? *World J Diabetes* 2022; 13(2): 126-128
URL: https://www.wjgnet.com/1948-9358/full/v13/i2/126.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i2.126

TO THE EDITOR

We read with interest the recent review, entitled "Effect of inflammatory bowel disease treatments on patients with diabetes mellitus", by Bower *et al*[1], which

Received: September 11, 2021
Peer-review started: September 11, 2021
First decision: December 4, 2021
Revised: December 9, 2021
Accepted: January 14, 2022
Article in press: January 14, 2022
Published online: February 15, 2022

P-Reviewer: Mastracci L, Wen XL
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

provided an excellent summary on the effects of different agents recommended for the treatment of inflammatory bowel disease (IBD) on glucose metabolism. These findings highlight the need for clinicians to consider the impact of IBD-related drugs on blood glucose control among IBD patients with diabetes, and also provide strong impetus to understand the potential mechanistic connection(s) between IBD and the onset of diabetes mellitus.

IBD refers to nonspecific chronic intestinal inflammatory conditions, including Crohn's disease (CD) and ulcerative colitis (UC). In the pursuit of the pathogenesis underlying IBD, 99 susceptibility loci/genes have been found to be related to IBD *via* genome-wide association studies. Interestingly, among those loci/genes, many are also associated with the risk of metabolic diseases, including type 1 and 2 diabetes[2]. A recent nationwide Danish cohort study has reported an increased risk of type 2 diabetes in patients with CD and UC, independent of glucocorticoid use[3]. Similarly, an elevated risk of type 1 diabetes was reported in pediatric patients with UC[4]. More recently, Jasser-Nitsche *et al*[5]. observed that in the German and Austrian population, children and adolescents with type 1 diabetes are at increased risk of IBD. These observations are, therefore, indicative of shared pathway(s) of pathogenesis between IBD and diabetes.

It is now widely appreciated that the gastrointestinal tract plays an important role in glucose homeostasis[6]. In recent years, there is mounting evidence that the gut microbial metabolites and their ensuing effects on the intestinal and systemic inflammation are associated with the occurrence and progression of diabetes; approximately 90% of type 2 diabetes is related to the disrupted gut microbiota, *i.e.* dysbiosis[7], a phenomenon also seen in IBD[8]. In the Danish cohort of IBD, specific abnormal microbial features are linked to the risk of type 2 diabetes[3]. Accordingly, dysbiosis may represent a common pathogenic factor of both IBD and dysglycemia.

Intestinal and metabolic homeostasis is also regulated by a number of gut-derived hormones, as a result of complex interactions between the ingesta and enteroendocrine cells. The incretin hormone glucagon-like peptide (GLP-1) is secreted from L-cells, which predominate in the distal small and large intestine[9]. GLP-1 regulates blood glucose metabolism *via* pleotropic actions, including stimulation of insulin secretion, suppression of glucagon secretion and energy intake, and slowing of gastric emptying [10]. In rodents, GLP-1 was reported to attenuate intestinal mucositis induced by chemotherapy[11]. In both patients with UC and CD and mice with colitis, the expression of GLP-1 receptor of intestinal biopsies was found to be reduced[12]; treatment with the GLP-1 receptor agonist, liraglutide, reduced levels of colonic inflammation in mice with colitis[12]. Accordingly, the reduction in the expression of dipeptidyl peptidase-4 - the enzyme that inactivates endogenously released GLP-1 – in the inflammatory bowel of patients with CD may have reflected a compensatory response of the gut to the development of inflammation[13].

Despite the reported association between the onset of IBD and diabetes, and the potential influence of IBD therapies on glucose metabolism, the common pathogenesis of IBD and diabetes remains elusive. Understanding the latter may provide novel therapeutic opportunities for these major chronic disorders.

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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 March 15; 13(3): 129-281

Published by Baishideng Publishing Group Inc

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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

PUBLICATION DATE
March 15, 2022

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 129-149

DOI: 10.4239/wjd.v13.i3.129

ISSN 1948-9358 (online)

REVIEW

Markers of insulin resistance in Polycystic ovary syndrome women: An update

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Zhao CF

Received: February 28, 2021
Peer-review started: February 28, 2021
First decision: July 15, 2021
Revised: September 14, 2021
Accepted: February 22, 2022
Article in press: February 22, 2022
Published online: March 15, 2022

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting 5%-10% of women of reproductive age. The importance of this syndrome lies in the magnitude of associated comorbidities: infertility, metabolic dysfunction, cardiovascular disease (CVD), plus psychological and oncological complications. Insulin resistance (IR) is a prominent feature of PCOS with a prevalence of 35%-80%. Without adequate management, IR with compensatory hyperinsulinemia contributes directly to reproductive dysfunction in women with PCOS. Furthermore, epidemiological data shows compelling evidence that PCOS is associated with an increased risk of impaired glucose tolerance, gestational diabetes mellitus and type 2 diabetes. In addition, metabolic dysfunction leads to a risk for CVD that increases with aging in women with PCOS. Indeed, the severity of IR in women with PCOS is associated with the amount of abdominal obesity, even in lean women with PCOS. Given these drastic implications, it is important to diagnose and treat insulin resistance as early as possible. Many markers have been proposed. However, quantitative assessment of IR in clinical practice remains a major challenge. The gold standard method for assessing insulin sensitivity is the hyperinsulinemic euglycemic glucose clamp. However, it is not used routinely because of the complexity of its procedure. Consequently, there has been an urgent need for surrogate markers of IR that are more applicable in large population-based epidemiological investigations. Despite this, many of them are either difficult to apply in routine clinical practice or useless for women with PCOS. Considering this difficulty, there is still a need for an accurate marker for easy, early detection and assessment of IR in women with PCOS. This review highlights markers of IR already used in women with PCOS, including new markers recently reported in literature, and it establishes a new classification for these markers.

Key Words: Markers; Insulin resistance; Polycystic ovary syndrome; Emerging markers; Impaired glucose tolerance

Core Tip: Diagnosing insulin resistance in Polycystic ovary syndrome is of crucial importance for better management and prevention of complications. Seeking of an easy-to-detect surrogate marker of insulin resistance represents a promising approach for maximizing treatment outcomes. This review highlights markers of insulin resistance already used in women with Polycystic ovary syndrome, including new markers recently reported in literature, and establishes a new classification of them.

Citation: Amisi CA. Markers of insulin resistance in Polycystic ovary syndrome women: An update. *World J Diabetes* 2022; 13(3): 129-149
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/129.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.129

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder, affecting 5%-10% of women of reproductive age.

According to the Rotterdam consensus[1], it is defined by at least two of the following abnormalities: oligo- and/or anovulation, clinical and/or biological hyperandrogenism, and polycystic ovaries.

The importance of this syndrome lies in the magnitude of associated complications[2,3]: Reproductive complications: menstrual dysfunction, infertility, hyperandrogenism, increased pregnancy complications, amongst others; Metabolic complications: insulin resistance and increased risk factors for type 2 diabetes (T2D) mellitus and cardiovascular disease (CVD); Oncological complications: Endometrial, ovarian and breast cancers; Psychological complications: Heightened anxiety, depression.

Insulin resistance (IR), the most common metabolic feature, is found in almost 35%-80% of PCOS women and is independent of body mass index (BMI) and body fat distribution[4-7].

IR is usually defined as a pathological condition characterized by a decreased responsiveness or sensitivity to the metabolic actions of insulin. It is an established predictor of a range of disorders. In women with PCOS, IR plays an important role in the development and persistence of this disorder[8,9] and is recognized to lead to many of the metabolic abnormalities associated with metabolic syndrome. PCOS patients with IR are likely to have chronic subclinical inflammation and impaired fasting plasma glucose levels, which in turn enhance the prevalence of the more atherogenic, low-density cholesterol (LDL-c) particles[10].

Given this high prevalence, the need for accurate screening of IR in women with PCOS is obvious.

Early recognition and management of IR in women with PCOS would offer important preventive measures[11].

MARKERS OF DIRECT MEASUREMENT OF INSULIN RESISTANCE IN PCOS WOMEN

Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp technique is the gold standard method for assessing beta-cell sensitivity in humans, quantifying the amount of glucose metabolized by the body following a controlled hyperglycemic stimulus[12]. It has been used in cross-sectional and prospective studies designed to test insulin sensitivity in women with PCOS[9,13-17] and the effect of interventions such as pharmacological treatment and lifestyle management (weight loss, weight gain, or diet changes)[18-26].

However, the glucose clamp is irrelevant for clinical practice. It is ill-suited for large-scale investigations because of extensive requirements in procedure, cost, time and technical expertise. Therefore, it is rarely used.

SURROGATE MARKERS OF INSULIN RESISTANCE IN WOMEN WITH PCOS

Since the glucose clamp is difficult to apply in large-scale investigations because of the chaotic procedure, surrogate markers are obviously needed. Over the years, simple markers have been developed and used in clinical practice. They include anthropometric and biological indices.

ANTHROPOMETRIC MARKERS

Anthropometry has been widely and successfully used for assessing health and nutritional risk. Several hundred papers have been published in the past five decades that have reported the close relation between different measures of body size and one or another cardiovascular risk factors[27-34]. Most of them have attempted to assess the robustness and nature of these associations. Thus, several measures have been described and proposed as surrogate markers of IR. Anthropometric markers could be divided into fat anthropometric markers and bone anthropometric markers. To date, bone anthropometric markers have been reported as the best anthropometric marker for insulin resistance.

Fat anthropometric markers

BMI: BMI is the ratio of weight to the square of height, initially described by Keys in 1976[35]. BMI has traditionally been the chosen method to measure body size in epidemiological studies. It is used as a measure of overall adiposity and a good marker of variability in energy reserves in individuals with a sedentary lifestyle[35-41]. The positive association between obesity and the risk of developing T2D has been repeatedly observed, both in cross□sectional studies and in prospective studies[36-40].

Over the years, BMI has been shown to be an accurate marker for detecting cardiovascular risk. BMI > 25 kg/m² is a major risk factor for a wide range of chronic diseases and metabolic abnormalities, including T2D and IR[35-40].

In women with PCOS, BMI is an independent predictor of IR[41-44]; however, it is not routinely used as a surrogate marker of IR. Indeed, since IR in PCOS is independent of body fat, it could not accurately be predicted by BMI in lean PCOS women. BMI correlates more closely with IR in overweight and obese women than in lean PCOS women.

Waist circumference: First suggested by Lean *et al*[45], to be more strongly associated with metabolic risk than BMI, the stronger positive association between cardiovascular risk factors and abdominal adiposity measured by anthropometric measurements of abdominal circumference has been confirmed by several studies[45].

According to the World Health Organization (WHO), waist circumference (WC) is measured at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest [46].

WC is an easy surrogate marker of visceral adiposity and is commonly used in daily medical practice to detect IR clinically. Increased visceral adiposity is associated with a range of metabolic abnormalities, including decreased glucose tolerance, reduced insulin sensitivity and adverse lipid profiles, which are risk factors for T2D and CVD. Moreover, WC is the core component of the definition of metabolic syndrome. It is specifically required for diagnosing metabolic syndrome according to the International Diabetes Federation and the 2003 Rotterdam consensus[1,47].

A considerable correlation has been found between WC and insulin resistance assessed by the hyperinsulinemic euglycemic clamp technique[48]. A wide WC > 80 cm has been shown to be associated with IR in women with PCOS[49]. Therefore, WC is now considered the most clinically relevant approach for the measurement of IR[50].

However, the use of WC, a fat anthropometric marker, for the assessment of IR in women with PCOS is limited because IR is independent of visceral adiposity[5,7,44]. Several studies have failed to show an association between WC and IR in lean women with PCOS [51]. WC could predict IR in overweight and obese PCOS women but not in lean PCOS women[5,51]. Consequently, it is not a good anthropometric surrogate marker for assessing IR in women with PCOS[44].

Waist-to-hip ratio: The waist-to-hip ratio (WHR) is an anthropometric index that combines waist and hip measurements. It is used as a measure of body fat distribution. According to the WHO, WHR is calculated as waist circumference divided by hip circumference[46]. WHR > 0.8 corresponded with a BMI overweight range of 25-29.9 kg/m².

Since it measures abdominal obesity, which in turn is attributed to the presence of visceral adipose tissue that promotes insulin resistance, WHR is used as a predictor of IR and metabolic risk. However, it has been described in several papers as a less accurate marker of adiposity that could predict cardiovascular and metabolic risk[27,44,52].

In PCOS assessment, its used has been practically abandoned[27,44,52].

Waist-to-height ratio: In the middle of the 1990s, the use of waist-to-height ratio (WHtR) was first proposed by Lee *et al*[32], for detecting abdominal obesity and associated health risks[53].

WHtR is calculated as waist divided by height.

Several studies have found a strong association of WHtR with cardiovascular risks. Indeed, it has been reported as the best anthropometric marker to assess T2D, metabolic syndrome, cardiovascular events, and altered blood pressure[53-57]. According to Ashwell *et al*[54], WHtR is one of the best alternative measures in predicting chronic diseases. In a systematic review comparing WC to WHtR, they found that the use of WHtR provided better results over WC for CVD outcomes, as well as for T2D and hypertension. In addition, Huxley *et al*[27] conducted a systematic review and meta-analysis of the anthropometric indices of cardiometabolic risk factors, involving 32 studies, to determine which of the

four indices (BMI, WC, WHR and WHtR) is the best discriminator of major cardiovascular risk factors. They found that measures of central obesity were superior to BMI as discriminators of risk of T2D, and therefore of IR. Huang *et al*[58], concluded that WHtR is one of the most representative marker to assess insulin resistance. The superiority of WHtR over BMI for detecting cardiovascular risk factors has been reported in a meta-analysis[59].

In women with PCOS, a few articles using WHtR as a marker are available. In a study conducted by Costa *et al*[60], in Brazilian women with PCOS, WHtR was the marker that presented significant positive correlations with the highest number of cardiovascular risk factors. They proposed the inclusion of this easily-measured parameter in the clinical assessment for the screening of women with PCOS and cardiovascular risk factors. Similarly, the results of a study by Gateva *et al*[61], indicated that both WHtR and WC, but not WHR, were good markers of adverse metabolic profiles in women with PCOS. More recently, Bhattacharya *et al*[62], suggested that WHtR could be used as an inexpensive and noninvasive screening tool for the early prediction of PCOS and IR among PCOS patients. Amisi *et al*[44], comparing several anthropometric markers, found that WHtR and WC showed similar performance but were less predictive of IR than wrist circumference.

Bone anthropometric markers

Wrist circumference: Wrist circumference (Wrc) was first proposed as a marker of insulin resistance in young obese people by Cappizzi *et al*[63]. His team was inspired by the findings of Karsenty *et al*[64], on the involvement of the bone system in glucose metabolism *via* osteocalcin (OC) effects on insulin[65-67]. Hyperinsulinemia has been associated with increased bone mass[68-70], and wide WrC has been associated with IR[71-74]. Esmaeilzadeh *et al*[75], found a positive correlation between WrC and PCOS status.

Amisi *et al*[44], showed that WrC is the best anthropometric marker known to date for the assessment of insulin resistance in women with PCOS. In their study, they reported a significantly higher correlation of nondominant WrC with IR than other anthropometric markers.

The novelty of WrC as a marker of IR is that it is based on the assessment of IR on bone, not on fat, as other anthropometric markers.

WrC is, to date, the only anthropometric marker that can assess IR in both obese and lean women. WrC is, consequently, the only useful clinical measure for assessing IR in lean women with PCOS. Given that most women with PCOS are insulin resistant, which is independent from fat and characterized by hyperinsulinemia, fat anthropometric markers are not suitable[44].

However, there are few publications on WrC as a marker of IR in women with PCOS.

BIOLOGICAL MARKERS

Markers using insulin and/or glucose

Oral glucose tolerance test: The oral glucose tolerance test (OGTT) is a frequently used index of glucose tolerance. It is commonly used in medical practice to detect IGT and T2D. Moreover, OGTT is the only means of identifying people with IGT[76]. The WHO recommends the test as a valid way to diagnose diabetes.

According to the WHO, the OGTT technique involves the oral administration of 75 g of glucose after 8 to 10 h of fasting. At 0, 30, 60 and 120 min following the oral glucose load, blood glucose levels are measured to determine how rapidly it is cleared from the bloodstream.

In PCOS women, the Androgen Excess Society in consensus with the European Society of Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM) have recently recommended a 2 h OGTT in all women with PCOS, with annual or biannual rescreening, depending on the risk factors[11,77,78]. The Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS consensus Workshop Group recommended screening for IGT and T2D when presented with the following conditions: hyperandrogenism with anovulation, acanthosis nigricans, obesity (BMI > 30 kg/m^2) in women with a family history of T2D or gestational diabetes mellitus[78].

However, OGTT provides useful information about glucose tolerance but not insulin resistance. In addition, it is more time-consuming and labor intensive to perform.

Glucose/insulin ratio: The glucose/insulin ratio (G/I) has long been employed as an index of IR[4,79-82].

It has been described by Legro *et al*[83], as a useful measure of insulin sensitivity in obese PCOS women and has both high sensitivity and specificity for detecting IR in women. In addition, the G/I ratio reflects profound peripheral IR and hepatic IR, which are found in obese women.

Furthermore, Quon confirmed the same in his editorial published in 2004, explaining how the G/I ratio correlates with insulin sensitivity in nondiabetic patients with PCOS[84]. In healthy subjects with normal fasting glucose levels, elevations in fasting insulin levels correspond to increased IR. Since fasting glucose levels are similar for all subjects, the G/I ratio is functionally equivalent to 1/insulin, which is a well-known proxy for insulin sensitivity. It decreases as a subject becomes more insulin

resistant and their fasting glucose rises[84].

However, the use of the fasting G/I ratio is limited in PCOS women with abnormal fasting glucose levels because, as demonstrated by Quon, this leads to erroneous results. Indeed, the G/I ratio is similar to 1/insulin in nondiabetic subjects, but it increases paradoxically in diabetic subjects and in PCOS women with abnormal glucose levels[84]. Consequently, the fasting G/I ratio has been considered a potentially flawed index of insulin sensitivity[84].

Fasting insulin: Numerous studies have investigated and proposed fasting insulin concentrations as the simplest index for assessing IR[85-87] because it has been shown to correlate well. High fasting insulin level in individuals with normal glucose tolerance has been found to reflect IR. Furthermore, high insulin concentrations presage the development of diabetes in the future[88].

In nondiabetic subjects with normal fasting glucose levels, the rise of fasting insulin levels corresponds to insulin resistance. In this population, insulin sensitivity, which decreases as subjects become more insulin resistant, can be substituted by 1/fasting insulin.

In women with PCOS, many authors have recommended fasting insulin as a simple office-based method to assess insulin resistance[77,89,90].

Recently, after comparing the prevalence of IR using published methods in a cohort of women with PCOS, Lunger *et al*[91], suggested the use of fasting insulin as a simple screening test. This can reduce the number of OGTTs needed to routinely assess IR in women with PCOS, as proposed by the Androgen Excess Society.

However, the use of fasting insulin for assessing IR in women with PCOS could be limited by a lack of adequate laboratories and the cost of insulin assays, especially in developing countries.

Minimal model analysis of frequently sampled intravenous glucose tolerance test: The frequently sampled intravenous glucose tolerance test (FSIVGTT) is an alternative method sought to simplify the clamp procedure. It provides information on both insulin sensitivity and β-cell function. The minimal model was developed by Bergman *et al*[92], in 1979 as a method to obtain an indirect measurement of insulin sensitivity or insulin resistance.

The standard technique of FSIVGTT includes multiple blood sampling for insulin and glucose. Baseline blood samples for insulin and glucose were taken at 15, 20, 25, and 30 min following the placement of an intravenous catheter. Glucose was then infused intravenously as a bolus over 1 min, followed by the extraction of blood samples for glucose and insulin measurements at 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160 and 180 min after the start of the glucose injection.

Plasma glucose and insulin concentrations collected during the test were subjected to minimal model analysis using the computer program MINMOD to generate an index of insulin sensitivity (Si).

Parameters derived from minimal model analysis have been found to correlate with those from euglycemic clamps[93].

Although FSIVGTT is minimally invasive and easier than euglycemic clamp, it is not suitable for large epidemiological studies. The complexity of the sampling procedure, the number of samples required and especially the corresponding higher cost make it unsuitable for clinical use.

Homeostasis model assessment: The Homeostasis Model Assessment (HOMA) is a method used to quantify insulin resistance from basal glucose and insulin levels, first described in 1985 by Matthews *et al*[94]. HOMA is a mathematical model of the relationship of insulin and glucose concentrations for a wide range of possible combinations of insulin resistance and β-cell function. It assumes the principle of interactions between β-cell deficiency, insulin resistance and fasting hyperglycemia. Consequently, any given decrease in insulin sensitivity and β-cell dysfunction is associated with fasting steady-state insulin and glucose concentrations. Using a computer-solved mathematical model of basal insulin and glucose interactions, the authors plotted a wide range of basal plasma insulin and glucose concentrations expected for possible combinations of insulin resistance and β-cell deficiency, to obtain the first model of HOMA. The early model was later updated using nonlinear solutions[95]. The approximating equation for insulin resistance has been simplified, and insulin resistance values can be derived from basal insulin and glucose concentrations as follows: HOMA-IR = insulin (mU/L) × glucose (mmol/L)/22.5.

The β-cell function is calculated as: HOMA β-cell = 20 x insulin (mU/L)/[glucose (mmol/L) - 3.5].

A strong linear correlation of HOMA-IR has been found with the euglycemic-hyperinsulinemic clamp [96,97]. However, HOMA-IR is determined from fasting concentrations of glucose and insulin. It provides an estimation of hepatic insulin sensitivity and could, therefore, assume the important limitation of identifying hepatic and peripheral insulin sensitivity. However, this is not the case in reality.

In women with PCOS, HOMA-IR has been used in various studies of distinct populations to assess insulin resistance[7,44,98-101]. Furthermore, the HOMA has proven to be a robust clinical and epidemiological tool for assessing IR. Similarly, HOMA β-cell has been used as a marker of basal insulin secretion by pancreatic β-cells[98].

In Sub-Saharan African women and in developing countries in general, HOMA-IR has been successfully used[7,44]. However, although the HOMA index has proven to be an accurate means to assess insulin resistance, it is difficult to perform in developing and low-resource countries because of

the cost of insulin measurements, as well as the lack of adequate laboratories and equipment.

Log (HOMA-IR): To more accurately reflect the physiology, other modifications have been made to the Homeostasis Model Assessment for insulin resistance (HOMA-IR). Using a computer program, log transformed HOMA-IR [Ln(HOMA-IR)] was obtained[96,102] and it correlates well with the eugylcemic clamp method[96].

Comparing Log(HOMA-IR) and HOMA-IR with the Minimal model, Log(HOMA-IR) correlated more strongly than HOMA-IR in nondiabetic subjects[103]. Log(HOMA-IR) has been found to be more convenient than HOMA-IR for the assessment of IR in mild to moderate diabetes and glucose intolerance. Moreover, Log(HOMA-IR) is a better predictor of insulin sensitivity than HOMA-IR[103].

Similar to HOMA-IR, log(HOMA-IR) has been extensively used in large epidemiological studies and in clinical research[103,104].

However, log(HOMA-IR) has been used in few studies for assessing IR in women with PCOS[42,105,106].

Fasting insulin resistance index: The fasting insulin resistance index (FIRI) was proposed by Duncan *et al*[107], in 1989.

FIRI is calculated as: FIRI = (glucose x insulin)/25.

However, in women with PCOS, FIRI has not been extensively used, similar to HOMA-IR[108,109].

Quantitative insulin sensitivity check index: Quantitative insulin sensitivity check index (QUICK) is an index of insulin sensitivity that provides a consistent and precise index of insulin sensitivity with better positive predictive power[110-111]. It is calculated from basal glucose and insulin concentrations obtained from a single fasting blood specimen. QUICKI is similar to HOMA and is simply its variation, as it interprets the data by taking both logarithms and the reciprocal of the fasting glucose-insulin product. Consequently, it is more accurate than HOMA in calculations over a wide range of insulin sensitivities.

QUICKI = 1/[log insulin (μU/mL) + log glucose (mg/dL)]

This formula implies that the lower the QUICKI value, the lower the insulin sensitivity.

QUICKI has been strongly correlated with measurements made by the euyglycemic clamp technique, especially in obese and diabetic subjects[112]. However, its performance was less satisfactory in subjects with normal glucose tolerance. Therefore, the revised QUICKI, which incorporates the fasting plasma free fatty acid concentration (FFA) into the equation, has been proposed[113-114]:

Revised QUICKI = 1/[log insulin (μU/mL) + log glucose (mg/dL) + log FFA (mmol/L)]

QUICKI has been shown to be appropriate and effective for use in large epidemiological or clinical research studies[111,115].

In a large meta-analysis of insulin-resistant subjects, Hanley *et al*[115], demonstrated that QUICKI is a simple surrogate index with the best positive predictive power for determining the development of diabetes.

In women with PCOS, QUICKI is among the most thoroughly evaluated surrogate indices for insulin sensitivity. It has been validated as a simple, inexpensive, useful, and minimally invasive surrogate index of insulin sensitivity[116-118].

Derived surrogate markers from OGTT

Some studies, carried out in other clinical conditions, suggested that surrogate indices derived from the OGTT could perform better than those obtained from fasting values[119-122].

Matsuda index: Additionally, called "the composite index", the Matsuda index was described by Dr Masafuni Matsuda and Prof Ralph DeFronzo in 1999. The Matsuda index, or the composite whole-body insulin sensitivity index (WBISI), is an index of IR derived from the OGTT that evaluates whole-body physiological insulin sensitivity. It is determined by insulin and glucose values obtained from the OGTT [120].

In women with PCOS, Rizzo *et al*[123], found that the Matsuda index correlates well with the HOMA-IR and QUICKI, indicating that it may be a reliable substitute in the detection of IR and subsequent intervention required to improve outcomes in women with PCOS. Ciampelli *et al*[90], observed that the Matsuda index obtained the best correlation coefficients with the euglycemic clamp in menopausal women.

Stumvoll index: Another index derived from the OGTT has been described by Stumvoll *et al*[121]. From demographic data (age, BMI, WHR), as well as insulin and glucose values obtained from the OGTT, they found a new index to predict insulin sensitivity and beta cell function.

However, in PCOS, only a few published studies have used the Stumvoll index[121,124-126].

In a recent study, Lewandowski *et al*[124], found that the correlation between various IR indices is highly variable when comparing surrogate methods based on fasting insulin and either fasting glucose (HOMA-IR and QUICKI) or triglycerides (McAuley Index), with IR indices derived from glucose and insulin during an OGTT (Belfiore, Matsuda and Stumvoll indices). They suggested that the clinical application of surrogate indices for the assessment of IR in PCOS must therefore be viewed with extreme caution[124].

Tosi *et al*[119], evaluated the performance of several surrogate markers of insulin resistance in identifying individual PCOS subjects with impaired insulin sensitivity, as defined by the euglycemic clamp, and found that all surrogate indices were highly correlated with hyperinsulinemic euglycemic clamp values. However, their ability to identify insulin-resistant individuals was limited in terms of sensitivity, especially in normal-weight subjects. ROC analysis showed similar performances of these indices (AUC values 0.782-0.817). They concluded that surrogate indices of insulin action show a low sensitivity in identifying insulin-resistant subjects, which causes many subjects to be erroneously diagnosed as insulin sensitive[119].

Avignon index: Avignon *et al*[127], also used OGTT values to try and develop another insulin sensitivity index. They compared sensitivity indices obtained from baseline fasting insulin and glucose levels (Sib), and at the end of the second hour of the OGTT (Si2h), a third insulin sensitivity index (SiM) was calculated by averaging Sib and Si2h. They observed that sensitivity indices obtained were useful to obtain a single test that could be used to determine both glucose tolerance and an estimate of insulin sensitivity.

In the study conducted in women with PCOS and menopausal subjects, which aimed to verify the validity of several indices of insulin sensitivity by comparing the data obtained by indices to those of the euglycemic clamp, Ciampelli *et al*[90] found that the best correlation with clamp studies was obtained with the Avignon Insulin Sensitivity Index in PCOS. The Matsuda index obtained the best correlation in menopausal patients[90].

Gutt index: In the search for a simple measure of insulin sensitivity, Gutt *et al*[122], also explored the use of OGTT values.

They devised a formula for an insulin sensitivity index, ISI (0, 120), that uses the fasting (0 min) and 120 min post oral glucose (OGTT), insulin and glucose concentrations. They found that ISI (0, 120) correlates well when applied prospectively in comparative studies, with the insulin sensitivity index obtained from the euglycemic hyperinsulinelic clamp[122].

In PCOS, Tosi *et al*[119], demonstrated the substantial pitfalls of derived surrogate indices, including the Gutt index, in identifying insulin-resistant individuals among PCOS women. Collectively, these indices showed a high PPV (90%-96%) but a low NPV (36%-45%). In other words, many subjects with insulin resistance were not recognized by any of these surrogate markers[119].

Insulinogenic index: The insulinogenic index (IGI) is derived from the OGTT to evaluate β-cell function.

IGI = [(30 min insulin - fasting insulin)/30 min glucose]

IGI is used to estimate the level of insulin secretion during glucose administration. The insulinogenic index has been commonly used during the first 30 min of the OGTT as a surrogate measure of first-phase insulin responses to a glucose challenge[128].

In women with PCOS, IGI is frequently used to express β-cell function[9,129-132].

Homa-M120: Morciano *et al*[133], first reported the aim of developing and validating a specific simple measure of insulin sensitivity using oral glucose tolerance test (OGTT) values for lean PCOS women because their cardiometabolic impairment is more frequently misunderstood. They showed that a temporarily delayed assessment of glucose and insulin concentrations during OGTT is more predictive of IR than a standard fasting evaluation, such as with HOMA-IR[133].

They then compared HOMA-M120 with other OGTT-derived indices and concluded that the 120-minute glucose and insulin evaluation (HOMA-M120) was the best IR index in lean PCOS women[133].

Song DK *et al*[126], made the same observation that lean women with PCOS, even when β-cell function is matched, showed higher values for HOMA-M120 but not HOMA-IR than matched controls.

Markers using lipid and lipoproteins

Abnormal lipid metabolism is one of the main characteristics of women with PCOS, with a prevalence of up to 70%[134-136]. Insulin resistance is closely associated with lipid disorders: elevated triglycerides (TGs), low-density cholesterol (LDL-c) levels and low high-density cholesterol (HDL-c) levels[136-142]. Increased serum concentrations of LDL-c and TG, as well as decreased HDL-c, are recognized as risk factors for cardiovascular disease[143-145]. Several epidemiologic studies have reported that lipid ratios are better predictors of atherosclerosis and cardiovascular disease than any other single lipid marker [144]. The superior ability of lipid ratios to predict the risk of cardiovascular disease than single lipid markers is of particular clinical interest.

Seeking a simple, effective and economic method to investigate IR, many researchers have suggested lipid ratios as surrogate indices[138-142].

Moreover, in PCOS patients, several studies have shown that the serum lipoprotein ratio has a significant positive correlation with IR and could be employed as a simple reliable indicator to determine IR[134-142,146].

TG/HDL-c: In overweight individuals with normal glucose tolerance, the TG/HDL-c ratio has shown the ability to identify IR with similar sensitivity and specificity to those of fasting plasma insulin concen-

tration. It has been proposed as a marker of insulin resistance[147]. Furthermore, low serum HDL-c combined with increased serum TG concentrations predicts the development of T2D[148].

In women with PCOS, Barrios *et al*[149], evaluated the relationship between the TG/HDL-c ratio and IR indices. They found that women with PCOS showed significantly higher TG/HDL-c ratios and HOMA-IR values, but lower QUICKI values. They proposed the TG/HDL-c ratio as a useful and practical method of assessing IR[149]. The same observation was made by Xiang *et al*[139]. The TG/HDL-c ratio seems to be the best index that directly correlates with insulin levels and can therefore be used as a marker of IR[138-140,149].

However, the problem with all markers using TG levels is that they could not be used efficiently in the African population because of the presence of TGs. Indeed, the Sub-Saharan African population presents what has been called the "TG paradox": Normal TG levels in the presence of IR[150]. This fact emphasized the previous need for a normal threshold of TG in the African population.

TC/HDL-c: Several epidemiologic studies have demonstrated that total cholesterol (TC)/HDL-c is a better predictor of atherosclerosis and cardiovascular disease than TC or HDL-c alone[144]. Furthermore, the TC/HDL-c ratio was shown to correlate negatively with insulin concentrations[151]. Subsequently, normal subjects with standard weight or overweight, as well as an increased TC/HDL-c ratio, have shown insulin resistance, increased TG concentrations, and hyperinsulinemia[152].

In women with PCOS, upon comparing the three lipid ratios commonly used as surrogate markers of IR (TG/HDL-c, TC/HDL-c, LDL-c/HDL-c), Xiang *et al*[139], found that the area under the ROC curve of TC/HDL-c was the largest, with the highest sensitivity and specificity. However, these findings were not confirmed in a similar study that reported the largest area under the ROC curve of TG/HDL-c[140].

LDL/HDL-c: Another index using lipoprotein is LDL/HDL-c ratio. It has also been found to correlate well with cardiovascular diseases.

In women with PCOS, it has been shown that LDL/HDL-c is an effective diagnostic marker for insulin resistance[139-140].

Emerging markers

Scientific evidence has disclosed strong influences between inflammatory mechanisms and IR. Some studies have shown that insulin resistance itself amplifies chronic inflammation[153]. PCOS is now recognized as a proinflammatory state associated with elevations in a number of circulating inflammatory mediators[154]. Therefore, it is not surprising that inflammatory markers have gained popularity in IR assessment, with several being proposed as surrogate markers of IR.

Interleukin-6: Interleukin-6 (IL-6), a major proinflammatory cytokine, has been shown to be closely associated with IR[155].

In women with PCOS, low-grade chronic inflammation has been reported and is involved in the pathogenesis of T2D and CVD[156]. However, conflicting results regarding IL-6 Levels in women with PCOS have been reported.

To evaluate IL-6 Levels in women with PCOS, a systematic review and meta-analysis were performed [157]. High levels of IL-6 have been reported to be related to IR. Interestingly, IL-6 levels have been reported to be high in both lean and obese women with PCOS. Indeed, IL-6 has been found to be related to IR and androgen levels but not to BMI.

However, Escobar-Morreale did not find statistically significant differences between PCOS and controls regarding IL-6 concentrations[154].

C-Reactive protein: C-Reactive protein (CRP) is one of the markers of systemic subclinical inflammation [158,159]. The relationship of CRP and several measures of IR has been described[160]. However, CRP alone could not predict IR.

It is well known that women with PCOS exhibit an elevation in circulating CRP that is independent of obesity[161]. Moreover, in a meta-analysis, circulating CRP was found to be 95% higher in women with PCOS than in controls[154]. This finding corroborates the existence of low-grade chronic inflammation in women with PCOS[156,161].

Nonetheless, in women with PCOS, elevation of CRP seems to be a PCOS effect rather than a result of IR. This fact limits its use as a good marker of IR.

Soluble CD 36: Soluble CD36 (SCD36) was initially described by Handberg *et al*[161], as a novel marker of IR. It has been found to be distinctly elevated in patients with IR and T2D[161].

In PCOS, a study conducted by Glintborg *et al*[162], reported that SCD36 correlated with measures of insulin sensitivity independent of central fat mass. Furthermore, pioglitazone treatment reduced SCD36 while improving insulin-stimulated glucose metabolism[162].

Nonetheless, more studies need to be conducted in PCOS to ascertain this association.

C3 complement: Recently, Muscari *et al*[163], reported a strong link between C3 complement (C3) and IR in an elderly population, independent of the components of metabolic syndrome. Some researchers have described the insulin-like properties of C3. Indeed, activation of C3 complement has been proven

to have insulin-like properties. It affects glucose transmembrane transport and promotes the synthesis of TG in adipocytes[164].

In PCOS, Yang *et al*[165], reported a strong association of serum C3 complement with insulin resistance. Lewis RD *et al*[166], observed a similar phenomenon. However, in a study conducted by Dehdashtihaghighat *et al*[167], such an association was not found.

Even so, this observation needs to be further investigated.

Ferritin: Ferritin, a major intracellular iron storage protein, has been proposed as a new marker of IR. High levels of ferritin have been associated with hyperinsulinemia and hypertriglyciridemia[168].

In PCOS women, elevated serum ferritin levels have been found to be associated with increased insulin resistance and the risk of diabetes in obese women but not in nonobese women[169]. Moreover, in both obese and nonobese PCOS women, higher serum ferritin levels have been correlated with a greater risk of hypertriglyceridemia.

In addition, elevated ferritin levels have been reported as a result of insulin resistance and hyperinsulinism but not reduced menstrual losses secondary to oligomenorrhea or amenorrhea[170, 171].

Nevertheless, more studies are needed to better clarify its applicability as a marker of IR in women with PCOS.

Adiponectin: Adiponectin is a protein produced by adipocytes with direct insulin sensitizing activity, plus vascular protective and anti-inflammatory effects. Adiponectin reduces glucose production by the liver and increases fatty acid oxidation in skeletal muscle. In addition to its antidiabetic effects, adiponectin possesses direct antiatherogenic properties[172,173]. In a variety of conditions frequently associated with IR, such as diabetes, hypertension and CVD, its plasma concentration has been found to be reduced[174,175]. Moreover, a reduction in high molecular weight (HMW) adiponectin levels, a fraction of adiponectin that is considered a potent mediator of insulin sensitivity, has been reported in IR states[176]. HMW is also decreased by testosterone[177]. It has recently been proposed that the ratio of HMW/total adiponectin, but not the absolute amounts of adiponectin, determines insulin sensitivity [178].

In women with PCOS, low serum adiponectin and HMW levels have been reported to be associated with IR[8,179-181]. It has been suggested that adiponectin may serve as the common denominator that connects obesity, IR and altered lipid metabolism in PCOS patients[177]. Furthermore, serum adiponectin levels have been suppressed in patients with both metabolic syndrome and IR. Consequently, the use of serum concentrations of adiponectin as a biomarker for insulin resistance has been suggested to distinguish PCOS patients at a higher risk of diabetes and cardiovascular morbidity [182].

However, the assumption that adiponectin is an intrinsic characteristic of IR in women with PCOS remains controversial. In addition, the effect of testosterone levels on adiponectin levels should be further investigated.

Tumor necrosis factor-α: Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine produced mainly by monocytes and macrophages. Several studies have shown a relation between TNF-α and IR in the general population[183].

In women with PCOS, multiple studies have demonstrated elevated levels of TNF-α[184,185].

TNF-α has been shown to impact ovarian function, including follicular development, ovulation, and corpus luteum regression[186]. Furthermore, it has been suggested that TNF-α promotes IR in women with PCOS and is implicated in the pathophysiology of PCOS[185].

However, Escobar-Morreale *et al*[154], in a meta-analysis cited above, found that TNF-α levels were not significantly different in women with PCOS compared to controls.

Therefore, the association of TNF-α and IR in women with PCOS remains controversial.

Glycosylated hemoglobin: Glycosylated hemoglobin (HbA1c) is the most common marker of chronic hyperglycemia and has long been considered the most practical approach used to review long-term glycemic control in diabetic patients. However, in 2010, the American Diabetes Association (ADA) included a glycated hemoglobin A1(c) (A1C) level as a component of diagnostic criteria of 'increased risk for diabetes'[187]. Since then, some researchers have conducted studies to examine the relationship of 'elevated A1C' (≥ 5.7%) with 'increased risk for diabetes' in women with PCOS to generalize its use as a screening test of prediabetes[188-192]. Indeed, increased HbA1c levels in the range of 5.7%-6.4% have been found to reflect IR or some component of metabolic syndrome[193].

However, the results reported in the current literature are controversial. A high prevalence of elevated A1C in nonobese patients with PCOS and an increased risk of elevated A1C have been associated with PCOS. Therefore, assessment of A1C as a useful new approach to screening for diabetes has been recommended[188,194]. Conversely, many studies do not support the recommendation that HbA1c can be used for the screening of prediabetes in women with PCOS because it failed to identify IR, though it was diagnosed in many PCOS patients by HOMA or fasting insulin levels[190,195].

Leptin: Leptin is an adipocyte-derived hormone that regulates a broad spectrum of homeostatic functions. It was the first adipokine to be identified[195,196]. One homeostatic function modulated by leptin is the regulation of insulin secretion by pancreatic β-cells and the regulation of insulin action and energy metabolism in adipocytes and skeletal muscle[197]. Leptin suppresses food intake and promotes energy expenditure mainly *via* its direct effects on hypothalamic neurons, and it is thus considered an antiobese hormone. Leptin levels decrease with fasting and increase with food intake[198,199].

A positive relationship between leptin, fat mass and BMI has been reported. Leptin levels are increased in obesity and significantly correlated with IR[200].

In women with PCOS, several prospective studies have confirmed that an increased leptin level is associated with insulin resistance and an elevated risk of obesity and diabetes[201-203]. Leptin has been found to have a strong positive correlation with HOMA-IR[204]. However, many studies failed to report any significant differences in serum leptin levels in women with PCOS when compared with age- and weight-matched controls[205-207]. The relationship between leptin and IR is thus still a matter of debate. Wang *et al*[208], did not observe significant differences in serum leptin between PCOS with IR and PCOS without IR. However, Yildizhan *et al*[202], observed an association between serum leptin levels and IR in young women with PCOS. Further investigation is needed to clarify the link between leptin and IR in women with PCOS.

Resistin: First found by Steppan *et al*[209], resistin is an adipokine that exerts an inhibitory effect on adipocyte differentiation and exerts resistance to insulin in mice. It has been suggested that resistin could be the potential link between obesity and diabetes[209,210]. Moreover, resistin seems to be an important adipokine that is involved in obesity, IR and PCOS[211].

However, these are hypotheses that need to be ascertained in humans. Data regarding the association between resistin and IR remain controversial. Many studies failed to recognize any association between resistin and IR[212-213], while a few studies indeed discovered a significant positive correlation[214-215].

In women with PCOS, conflicting results have also been reported[216-218]. Munir *et al*[216], reported increased concentrations of serum resistin levels in women with PCOS in comparison to controls. However, no significant difference was found in circulating resistin levels between PCOS and controls in most studies[217,218].

Vaspin: Elevated serum and omental adipose tissue levels of visceral adipose tissue-derived serine protease inhibitor (vaspin) in overweight PCOS women and *ex vivo* regulation of vaspin, predominantly by glucose, were reported, for the first time, by Tan *et al*[219]. A similar result was found by Dogan *et al*[220]. However, Franik G *et al*[221], did not observe correlations between plasma vaspin levels and serum glucose and insulin concentrations or HOMA-IR values.

Apelin: Apelin is a peptide expressed in several organs and in visceral and subcutaneous tissues[222].

Controversial results have been reported by different authors. Several authors reported elevated apelin, while others reported low serum levels of the same[223-228]. Polak *et al*[8], in their recent review of the literature, concluded that discrepant findings among the published studies may be attributed to the differences in ethnicity, age, study design, sample size, genetic characteristics of populations, and assessment methodology. Further studies are necessary to elucidate the role of apelin in insulin resistance in PCOS.

Copeptin: Copeptin, a vasoactive peptide, has been reported to play an important role in CVD and metabolic disorders. Enhanced copeptin levels in PCOS patients are positively associated with fasting insulin, HOMA-IR, androgenic profile, triglycerides and carotid intima media thickness, indicating that copeptin may play an important role in cardiometabolic consequences in PCOS[8,229-231].

However, to date, few studies have been performed to assess copeptin as a marker of IR in women with PCOS.

Further data from large-scale longitudinal studies are required for its validation.

Irisin: Irisin is a myokine identified as a new marker of IR[8,232-234].

In PCOS, a significant positive correlation between circulating irisin, IR and dyslipidemia has been found. Li *et al*[233], demonstrated that irisin levels were significantly higher in PCOS subjects than in controls, as well as in overweight and obese patients than in lean women. Similar results were obtained by Li *et al*[234].

Further studies are necessary to confirm these findings.

Zinc-α2-glycoprotein: Zinc-α2-glycoprotein (ZAG) has been proposed to play a role in the pathogenesis of insulin resistance[235].

In women with PCOS, Lai *et al*[236], found that women with PCOS and high ZAG had fewer metabolic syndrome, IGT and polycystic ovaries than those with low ZAG. Taken together, circulating ZAG levels are reduced in women with PCOS. They concluded that ZAG may be a cytokine associated with insulin resistance in women with PCOS[236,237]. Pearsey *et al*[238], arrived at a similar conclusion.

Zheng *et al*[238], performed a study to investigate changes in ZAG levels after exenatide or metformin treatment. The results showed that circulating ZAG was significantly lower in women with PCOS than in healthy women. After 12 wk of exenatide or metformin treatment, there were significant increases in circulating ZAG in both treatment groups[238].

Therefore, more research is needed before robust conclusions can be drawn[8].

Plasminogen activator inhibitor-1: Numerous studies have reported the association between IR and plasminogen activator inhibitor-1 (PAI-1), a glycoprotein involved in the coagulation system[8,239-241].

PAI-1 has been found to be linked to insulin resistance in PCOS subjects[8,239-242].

Further data from large-scale longitudinal studies are required for its validation.

CONCLUSION

This article is an attempt to summarize existing markers of IR and their usefulness in women with PCOS. There is no recommended screening method for assessing IR in women with PCOS despite evidence of the high prevalence of this metabolic disturbance.

A host of methods have been described for assessing insulin resistance. Each method has its own merits and disadvantages.

The euglycemic clamp remains the gold standard for direct measurement of insulin sensitivity.

Concerning anthropometric surrogate markers, wrist circumference could revolutionize the assessment of IR in women with PCOS if validated through large-scale studies.

Regarding biological surrogate markers, HOMA-IR is the best and extensively validated marker.

Biological markers using lipids and lipoproteins are inconsistent in the Sub-Saharan African population and hence in Sub-Saharan African PCOS women.

Conflicting data concerning emerging markers in women with PCOS limit their use in the clinical setting.

Finally, an easy-to-detect marker for assessing IR in women with PCOS is urgently required.

FOOTNOTES

Author contributions: Chantal Amisi wrote the manuscript.

Conflict-of-interest statement: The author declares that she has no conflicts of interest.

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S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

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World Journal of Diabetes

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World J Diabetes 2022 March 15; 13(3): 150-160

DOI: 10.4239/wjd.v13.i3.150

ISSN 1948-9358 (online)

REVIEW

Efficacy of probiotics on the modulation of gut microbiota in the treatment of diabetic nephropathy

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kobyliak N, Xu PF

Received: March 5, 2021
Peer-review started: March 5, 2021
First decision: May 3, 2021
Revised: May 21, 2021
Accepted: February 11, 2022
Article in press: February 11, 2022
Published online: March 15, 2022

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Abstract

Diabetic nephropathy (DN) is a major cause of end-stage renal disease, and therapeutic options for preventing its progression are insufficient. The number of patients with DN has been increasing in Asian countries because of westernization of dietary lifestyle, which may be associated with the following changes in gut microbiota. Alterations in the gut microbiota composition can lead to an imbalanced gastrointestinal environment that promotes abnormal production of metabolites and/or inflammatory status. Functional microenvironments of the gut could be changed in the different stages of DN. In particular, altered levels of short chain fatty acids, D-amino acids, and reactive oxygen species biosynthesis in the gut have been shown to be relevant to the pathogenesis of the DN. So far, evidence suggests that the gut microbiota may play a key role in determining networks in the development of DN. Interventions directing the gut microbiota deserve further investigation as a new protective therapy in DN. In this review, we discuss the potential roles of the gut microbiota and future perspectives in the protection and/or treatment of kidneys.

Key Words: Diabetic nephropathy; Short chain fatty acids; Superoxide dismutase; Reactive oxygen species; D-amino acids; Gut microbiota; Diabetes mellitus; Renal disease

Core tip: Evolving evidence suggests that the gut microbiota may play a key role in the development of diabetic nephropathy (DN). Interventions aimed at the gut microbiota deserve further investigation as a novel protective therapy in DN. We review the potential roles of the gut microbiota in the protection of kidneys and in the development of DN.

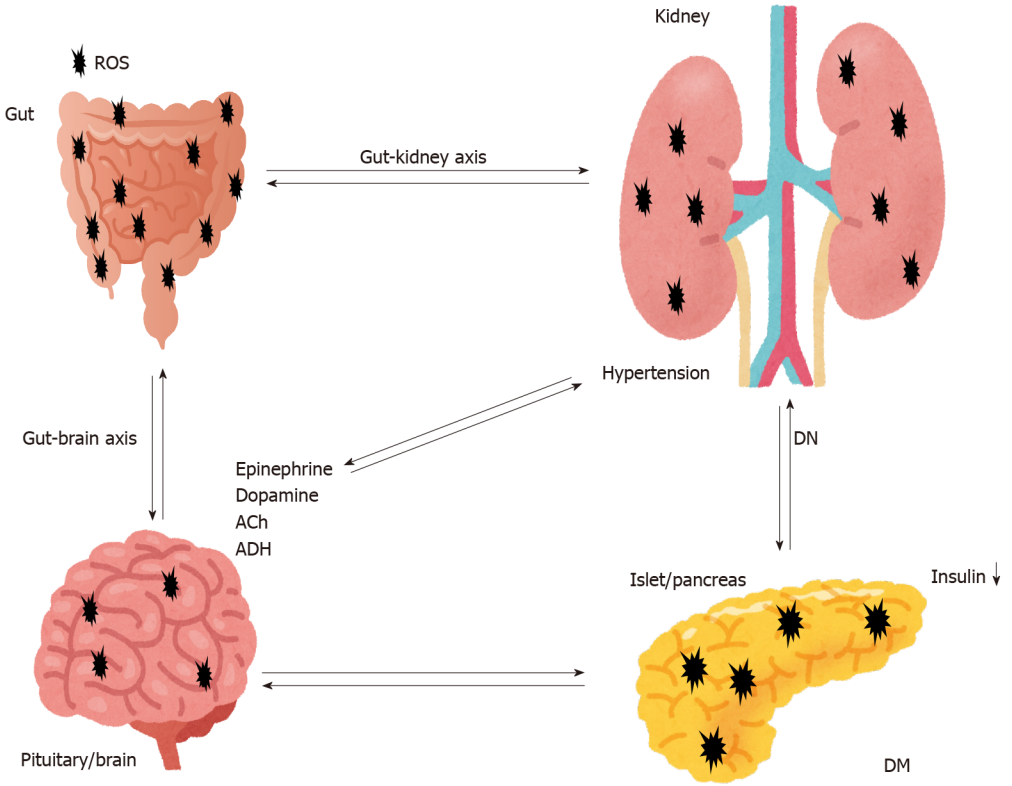
Citation: Nagase N, Ikeda Y, Tsuji A, Kitagishi Y, Matsuda S. Efficacy of probiotics on the modulation of gut microbiota in the treatment of diabetic nephropathy. *World J Diabetes* 2022; 13(3): 150-160
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/150.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.150

INTRODUCTION

Diabetic nephropathy (DN) is a chronic disorder occurring in nearly 40% of patients with diabetes[1]. DN is an important cause of end-stage renal disease and a micro-vascular complication of diabetes mellitus (DM)[2,3]. Some dietary factors might be involved in the increase in renal failure in association with DM, showing that the number of patients with DN and/or DM has been increasing in Asian countries because of westernization of dietary lifestyle[2,3]. Pathogenesis of DN may be multifactorial and complex. Early DN has no noticeable clinical symptoms, however, hyperglycemia may be a significant risk factor for DN and/or DM[4]. Sustained elevated blood glucose could lead to changes in the downstream transcription factors and/or gene expression in kidney glomerular cells[5]. Kidney fibrosis and albu-minuria are key pathological processes of the advanced stage of DN[6], but oxidative stress and/or inflammation may also be important mechanisms for the pathogenesis of DN[7]. In general, oxidative stress and inflammatory responses are almost not distinct, because one reaction would intensify the other pathogenesis. Both DM and chronic kidney disease (CKD) may have a common pathophysiological mechanism within a chronic inflammatory state and/or oxidative stresses [8]. Among them, high levels of reactive oxygen species (ROS) could induce inflammatory cytokines in the kidney[9], which might accelerate the development of DN. Inflammation of the kidneys can lead to proteinuria and/or persistent hypertension, which can proceed to renal failure. Hence, successful treatment of the microcirculation in patients with DN has become a superior strategy for the prevention of DN. This reasonable treatment should be discovered immediately. Recently, it has been shown that pathogenesis of DN is associated with certain gut microbiota[10]. The importance of probiotics is widely recognized in various diseases. Besides, studies have shown that crosstalk between host and microbiota might be relevant pathologically in patients with DN[11]. For example, alterations in the gut microbiota are associated with the development of proteinuria[12], and type 2 DM[13]. Changes to the gut microbiota have also been reported in DM and DN[14]. The gut microbiota might well communicate with the kidneys, and the collapse of this relationship might result in the development of renal dysfunction. Accordingly, the gut microbiota could be an important defense against the pathogenesis of kidney disease. Dietary lifestyles have radically changed over the last century in developed countries, and are characterized by reduced dietary fiber and/or increased high-fat consumption[15]. Hence, the changes could be linked to alteration of gut microbiota[16]. Abnormal intestinal metabolites and disruption of the intestinal barrier owing to the gut dysbiosis might facilitate harmful substances produced in the gut entering the circulatory system[17]. These situations allow us to hypothesize that dietary changes could lead to a microbiome that modifies positively the threshold and/or the speed of developing DN and/or DM.

GUT-KIDNEY AXIS IN THE PATHOGENESIS OF DN

Although the significance of the gut microbiota has yet to be completely determined, it is obvious that an intricate symbiotic relationship might exist between host and microbe. In addition, the interaction has recently attracted interest in the study of the pathogenesis of various disorders. The human body holds numerous bacterial and/or microbial cells; the majority of which exist in the gut[18]. The microbiota is a complex community of more than 100 trillion cells in healthy human intestines[19]. The normal gut microbiota could protect the kidney, whereas gut dysbiosis of the microbiota could facilitate kidney disorders[20]. Furthermore, alterations in the microbiota are gradually being linked to the development of various other diseases such as inflammatory bowel disease, cancer, psychiatric disorder, and cardiovascular disease[21]. The gut-kidney axis could additionally affect metabolic and/or immune pathways in addition to the related diseases[22]. The gut-kidney axis is largely mediated by metabolites produced by the gut microbiota, which might regulate physiological function of several organs including the brain, pancreas, adrenal glands, kidneys, *etc.* (Figure 1). For example, components of the immune system might have a key role with cytokines in communication between the gut and kidneys [23]. Furthermore, crosstalk between the metabolic and immune pathways has a significant role in keeping a good balance in the kidneys[23]. Intestinal responses to inflammation and/or infections are intricate. If microbiota-immune pathways overstimulate tolerance to some inflammation, greater inflammation may accelerate progression of renal disease and/or its complications. Accordingly, gut dysbiosis has frequently been associated with progression of many kidney diseases[24]. In addition, accumulation of uremic toxins, which are derived from dietary metabolism in the gut and/or liver, has distinct effects



DOI: 10.4239/wjd.v13.i3.150

Figure 1 Representation of the pivotal role of gut-kidney axis crosstalk with the brain and the pancreas in the pathogenesis of diabetic nephropathy. Hypothetical image of the pathogenesis pathway for diabetic nephropathy (DN). Sympathetic activation is a common feature in disorders of the brain as well as gut and kidneys. The brain is responsible for sympathetic outflow contributing to an increase in blood pressure and pathogenesis of the gut and kidneys. Dysbiosis in the gut results in an imbalance of intestinal homeostasis. Pathological events in the brain, pancreas, adrenal glands, gut and kidneys significantly contribute to the development of hypertension and DN. Note that some critical pathways such as inflammation pathway have been omitted for clarity. Ach: Acetylcholine; ADH: Antidiuretic hormone; ROS: Reactive oxygen species; DM: Diabetes mellitus; DN: Diabetic nephropathy.

on the kidneys. For example, the increase in urea increases its influx into the bowel lumen from epithelial cells, where it is hydrolyzed by gut microbiota urease to ammonia[25]. Subsequently, ammonia byproducts may increase the bowel pH, leading to the severe mucosal damage[26]. Accumulation of the uremic toxins in combination with inflammation may also increase the risk of renal disease [27]. Therefore, key factors in kidney disease are function of the gut microbiota and/or the action of gut dysbiosis. Inflammatory bowel disease and DM are indeed multifactorial diseases, and both are chronic diseases associated with increased risk of various diseases including cardiovascular disease, which indicates that the gut is associated with host physiological functions[28]. Interestingly, the prevalence of inflammatory bowel disease in adults with type 1 DM is higher compared to that of nondiabetic controls [29]. It is plausible that the gut-kidney axis might be involved in the pathogenesis of inflammatory bowel disease and DM. Similarly, the gut microbiota may be involved in the damage of other organs, hence targeting the gut microbiota could represent a future therapeutic approach in various diseases. However, the potential impact of gastrointestinal-related disorders on the development and/or progression of DN remains to be elucidated.

LEVELS OF SHORT-CHAIN FATTY ACIDS, ROS, AND D-AMINO ACIDS MAY BE INVOLVED IN THE DEVELOPMENT OF DN

Diabetic model mice fed with a high-fiber-diet are less likely to develop DN compared with diabetic control mice fed with a no-fiber diet[30]. High-fiber diet might decrease the expression of genes encoding inflammatory cytokines related to DN[30]. In general, fibers positively improve the dysbiosis of microbiota with promoting the production of short chain fatty acids (SCFAs) (including butyrate, acetate and propionate) in gut microbiota[31], which might also increase the production/release of cytokines and/or chemokines[32]. In addition, SCFAs are able to inhibit intestinal inflammation and/or oxidative stress[33]. Major SCFAs (acetate, propionate and butyrate) are derived through glycolysis of glucose to pyruvate or acetyl-CoA. The SCFAs regularly induce glucagon-like peptide 1 secretion through stimulation of a G-protein-coupled receptor (GPCR)[34]. Gut microbiota in older people may

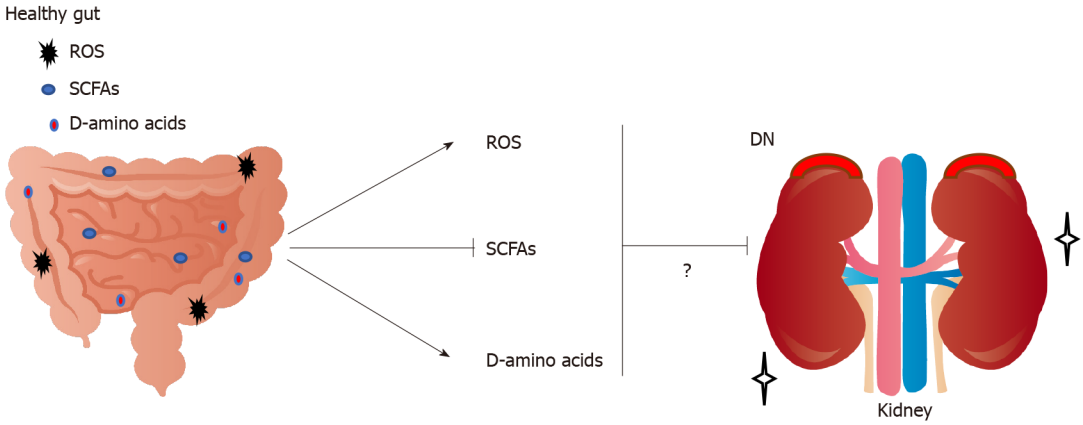
weaken SCFA production[35]. Those SCFAs have various effects on endocrine cells in gut *via* the GPCRs such as G-protein-coupled receptor (GPR)43 or GPR109A[36]. SCFA-treated diabetic mice have been shown to be protected from nephropathy, suggesting that SCFAs protect renal cells from injury by oxidative stress in DN[37]. It has been shown that butyrate, one of the SCFAs produced by gut microbiota, plays a protective role in DN, which contributes in various physiological processes predominantly by inhibiting histone deacetylases (HDACs)[38]. In addition, providing sodium butyrate has been shown to protect renal cells from oxidative damage and/or apoptosis in type 2 DN mice[39]. Consistently, sodium butyrate has inhibited high-glucose-induced apoptosis of tubular epithelial cells in normal kidneys[40]. Sodium butyrate also lowers plasma glucose and nuclear factor-B expression in the kidneys and attenuates kidney injury[41]. In experimental mice, suppression of HDACs by sodium butyrate may explain the decrease in apoptosis in the kidneys[42]. HDACs can regulate cell proliferation, migration and apoptosis, which are organized by a family of enzymes important for chromatin remodeling, keeping a dynamic balance with histone acetyltransferases in expression of several genes [43]. Valproate, an HDAC inhibitor, has also been shown to decrease renal injury and/or renal fibrosis [44].

The signaling pathways triggered by hyperglycemia appear to have a pivotal role in diabetic complications due to the production of ROS and/or additional oxidative stress, which finally leads to apoptotic cell death in various tissues[45]. ROS includes superoxide anions, hydroxyl free radicals, and hydrogen peroxide[46]. The mitochon-drial electron transport chain is considered a major endogenous source of ROS[47]. Production of excess ROS leads to increased membrane permeability and serious cellular damage[48]. Such overproduction of ROS links to the pathological condition of altered metabolic pathways in the kidneys and disturbed renal function known as nephropathy[49]. Once ATP synthesis is dysregulated in this hyperglycemic situation, it can result in excess production of ROS, which leads to kidney failure[50]. Furthermore, high glucose exposure with excessive ROS can lead to renal podocyte apoptosis in experimental DN[51]. Antioxidants including ubiquinone (also termed coenzyme Q10), ascorbic acid, and resveratrol have been tested in animal models of kidney diseases with some evidence of therapeutic benefits[52]. Epidemiological studies have also found an association between high levels of ROS and risk of DN[53]. Therefore, downregulation of ROS and/or oxidative stress might have a crucial role in regulating diabetic complications. Besides, ROS have been revealed to function as second messengers in several signal transduction pathways[54,55].

Studies have shown the clinical significance of D-amino acids in several kidney diseases[56]. For example, the combination of blood level and urinary dynamics of D-serine effectively separates CKD from non-CKD[57]. D-amino acids in body fluids are also a promising early detection marker for kidney disease[58]. However, excess D-serine can cause kidney damage in rats[59]. In this case, it has been shown that D-serine administration can initiate extensive necrosis in renal proximal tubules[59]. In contrast, administration of D-alanine does not induce kidney injury[60]. Furthermore, protective effects of low-dose D-serine have likely been shown to suppress renal damage, which may promote the hypoxia-mediated proliferation of tubular epithelial cells[61]. In addition, D-cysteine administration can also protect the kidneys from ischemia-reperfusion injury, which might be useful to treat various renal diseases[62]. D-aspartate plays a role during development and neurogenesis[63]. D-aspartate treatment might produce favorable effects during demyelination and remyelination in the nervous system[64]. Furthermore, the ovary-inducing activity of D-tryptophan is more effective than that of L-tryptophan [65]. These data suggest that D-amino acids have both beneficial and harmful effects on tissue development and/or tissue-protection (Figure 2).

GUT MICROBIOTA COULD CONTRIBUTE TO HEALTHY KIDNEYS

Carbohydrates are metabolized by gut bacteria into monosaccharides and oligosaccharides, and they could be fermented into SCFAs. As shown above, SCFAs are one of the primary end products of gut fermentation that have considerable effects on host physiology. SCFAs can act as signaling molecules between the gut microbiota and host, and may have a protective effect on the renal function of patients with CKD. In particular, butyrate improves the intestinal barrier and reduces lipopolysaccharide influx into the blood, which could attenuate progression of DN[66]. We provide here a perspective of gut-kidney axis applied in search of renal disease management associated with the gut microbiome, which may theoretically be beneficial for future treatment of DN. Diet is known to be an essential regulator of gut microbiomes[67]. Many studies have confirmed the association between nutrition and the human microbiome in maintaining human health, suggesting significant roles of bacterial metabolites in both health and disease[68]. Trillions of bacteria present in the intestinal and colon lumina constitute the human gut microbiota[69]. Dietary intake could control microbiota whose fermentation may produce various metabolites including SCFAs[70]. The metabolites might additionally regulate the growth of pathogens by competing for of nutrients. For example, parenteral nutrition has been associated with a change in the microbiota, altering SCFA production, and inducing gut mucosal atrophy[71]. The SCFAs made by the healthy gut microbiota have anti-inflammatory properties, including proliferation of regulatory T cells[72,73]. In addition, a significant role for regulatory T cells has been revealed in type 2



DOI: 10.4239/wjd.v13.i3.150

Figure 2 Implication of increased short-chain fatty acids, decreased reactive oxygen species, and increased D-amino acids derived from gut in the renal protection and/or exacerbation against the progression of diabetic nephropathy. Arrowheads mean stimulation and/or progression, whereas hammerhead represents inhibition. Note that some critical pathways including hormonal regulation have been omitted for clarity. SCFAs: Short-chain fatty acids; ROS: Reactive oxygen species; DN: Diabetic nephropathy.

diabetes for protection against DN[74]. In addition, SCFAs have favorable effects on β cells, potentiating glucose-stimulated insulin release and/or maintaining β-cell mass through inhibiting apoptosis[75]. Furthermore, propionate, has been shown to prevent adipogenic differentiation of specific stem cells [76].

Many studies have emphasized the relationship between the gut microbiota and oxidative stress[77]. In general, ROS production has a defense mechanism that could elicit cytotoxicity against several pathogens then reduce the burden of infection[78]. Redox signaling is also found in response to microbial signals *via* the gut epithelial NADPH oxidase 1[79]. Therefore, microbial ROS might rigorously control signaling processes for appropriate immunity and/or the gut barrier[80]. Numerous bacterial species of the microbiota can reduce mitochondrial ROS production[81]. For example, microbial products can upregulate the activity of superoxide dismutase, which results in reduced ROS levels and then decreased cellular apoptosis[82]. In addition, microbial excess ROS might disturb other important pathways of host cells, suggesting that ROS-mediated signaling can regulate various cellular processes in order to keep the host healthy[83]. Epithelial cells may also exhibit increased ROS production in response to several harmful bacteria[84]. In the gut, epithelial appropriate ROS production in response to the gut bacteria may play a signaling role in the host[85]. It is likely that there are many ROS-sensitive important enzymes that could be affected by alterations in the gut redox conditions.

Finally, the gut microbiota have the largest genetic capacity to metabolize D-amino acids that are utilized as nutrients to support bacterial growth to regulate spore germination[86]. Therefore, one possible source of D-amino acids in mammals may be their gut microbiota. In general, many bacterial species encode racemases that convert L-amino acids to D-amino acids[87]. For example, D-alanine production is associated with a relative abundance of bacterial species with racemases such as those of *Enterococcus* and *Lactobacillus* in the gut microbiota[88]. Different bacterial species may produce distinct profiles of D-amino acids[89]. Higher D-amino acids levels have been related to the gut microbial mass [90]. Oral intake of a peptide containing specific D-amino acids may reverse the diabetes-associated pathological alterations in the kidneys[91] (Figure 2). Noteworthy differences in the microbiota composition have been discovered in patients with kidney disease compared with healthy controls[92]. Consequently, treatment options for DN should include dietary therapy affecting the gut microbiota. Therapeutic interventions would nevertheless represent a potential target of the microbiota for prevention and/or treatment of DN.

CONCLUSION

New therapies for DN are emerging. One method that may affect the gut microbiota composition is fecal microbiota transplantation (FMT) (Figure 3). The beneficial effects of the transplantation are dependent on the host responses, however, which may provide a potential treatment strategy for type 2 diabetes[93]. In particular, transplantation of *Faecalibacterium prausnitzii* (*F. prausnitzii*) could restore the intestinal structure, which might be used as a potential therapeutic approach against inflammation as well as diabetes[94-96]. Furthermore, *F. prausnitzii* may serve as a diagnostic and therapeutic biomarker for the use of FMT[97]. The potential role of the gut microbiota has been hypothesized to modulate renal function in experimental DN murine models[98]. Through FMT, the role of the gut microbiota and its

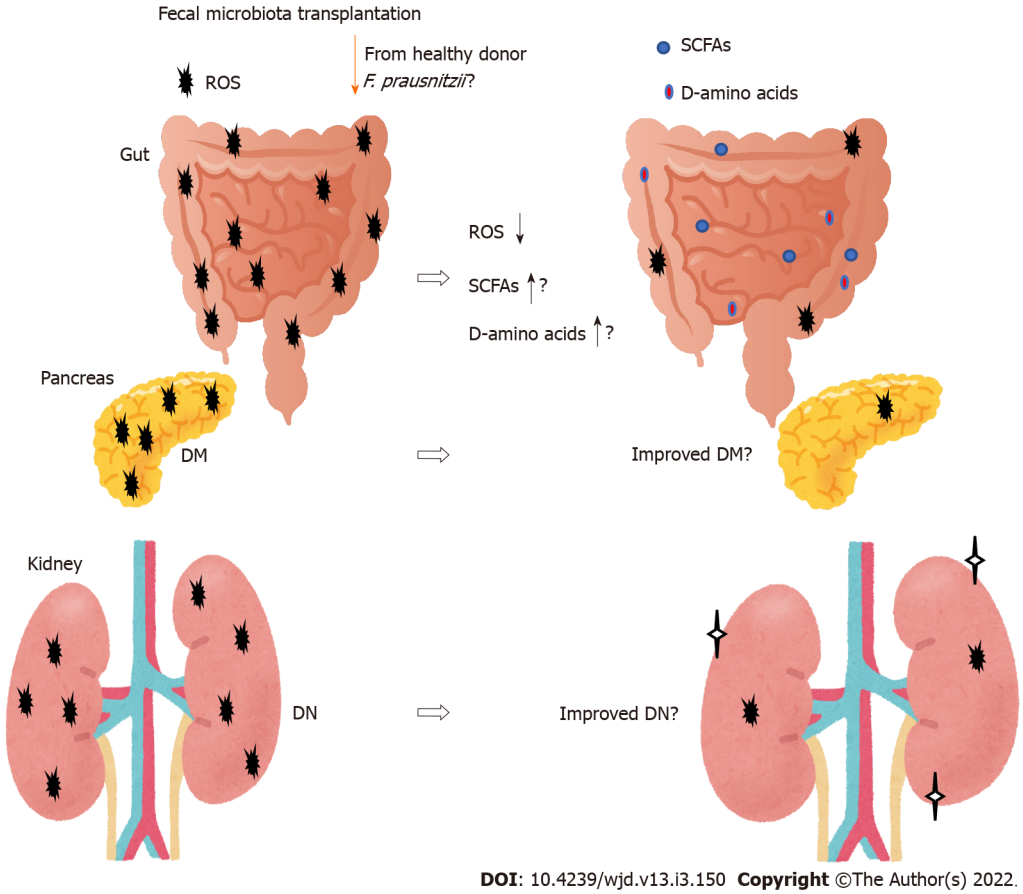


Figure 3 The gut microbiota could contribute to the favorable production of short-chain fatty acids, reactive oxygen species and D-amino acids against progression of diabetic nephropathy. Fecal microbiota transplantation consists of fecal microbiota infusion from a healthy donor to a recipient, which has been likely more successful than conventional therapy for diabetic nephropathy. Note that some critical events such as cytokine-induction have been omitted for clarity. SCFAs: Short-chain fatty acids; ROS: Reactive oxygen species; DN: Diabetic nephropathy; DM: Diabetes mellitus.

SCFA production have been verified in the treatment of DN. Therefore, administration of prebiotics and/or probiotics should individually be tailor-made to prevent and/or cure chronic diseases such as DN. For example, acetate produced by certain gut microbiota reprogramming has been shown to contribute to the tubulointerstitial injury of DN, suggesting that gut microbiota might be a new strategy for DN treatment[99]. Furthermore, FMT from healthy donors considerably attenuates glomerular injury with podocyte improvement in diabetic rats[100].

The above-mentioned topics are only just being explored in preclinical research, suggesting that further studies are required. Owing to a lack of treatments, DN has been a public health concern. Although it is untimely to draw definitive conclusions about the clinical usefulness of microbiota-based treatment strategies for DN, modulation of gut microbiota is an exciting frontier in kidney research. It is clear that intensive evaluation of preclinical studies is necessary to find further insights. In addition, long-term studies are also necessary to clarify the detailed effects of probiotic treatment in the management of DN. A healthy lifestyle with a balanced familiar diet is now one of the main recommendations.

FOOTNOTES

Author contributions: Each author has participated sufficiently in the work of drafting the article and/or revising the article for important rational content; all authors give final approval of the version to be submitted.

Conflict-of-interest statement: The authors declare that they have no competing financial interests.

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S-Editor: Wang JJ
L-Editor: Kerr C
P-Editor: Wang JJ

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Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 161-184

DOI: 10.4239/wjd.v13.i3.161

ISSN 1948-9358 (online)

REVIEW

Protective effects of physical activity against health risks associated with type 1 diabetes: "Health benefits outweigh the risks"

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Plagens-Rotman K, Shalaby MN

Received: September 11, 2021
Peer-review started: September 11, 2021
First decision: December 4, 2021
Revised: December 8, 2021
Accepted: February 20, 2022
Article in press: February 20, 2022
Published online: March 15, 2022

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Abstract

The magnitude of diabetes mellitus (DM) has increased in recent decades, where the number of cases and the proportion of the disease have been gradually increasing over the past few decades. The chronic complications of DM affect many organ systems and account for the majority of morbidity and mortality associated with the disease. The prevalence of type 1 DM (T1DM) is increasing globally, and it has a very significant burden on countries and at an individual level. T1DM is a chronic illness that requires ongoing medical care and patient self-management to prevent complications. This study aims to discuss the health benefits of physical activity (PA) in T1DM patients. The present review article was performed following a comprehensive literature search. The search was conducted using the following electronic databases: "Cochrane Library", Web of Science, PubMed, HINARI, EMBASE, Google for grey literature, Scopus, African journals Online, and Google Scholar for articles published up to June 21, 2021. The present review focused on the effects of PA on many outcomes such as blood glucose (BG) control, physical fitness, endothelial function, insulin sensitivity, well-being, the body defense system, blood lipid profile, insulin resistance, cardiovascular diseases (CVDs), insulin requirements, blood pressure (BP), and mortality. It was found that many studies recommended the use of PA for the effective management of T1DM. PA is a component of comprehensive lifestyle modifications, which is a significant approach for the management of T1DM. It provides several health benefits, such as improving BG control, physical fitness, endothelial function, insulin sensitivity, well-being, and the body defense system. Besides this, it reduces the blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality. Overall, PA has significant and essential protective effects against the health risks associated with T1DM. Even though PA has several health benefits for patients with T1DM, these patients are not well engaged in PA due to barriers such as a fear of exercise-induced hypoglycemia in particular. However, several effective strategies have been identified to control exercise-induced hypoglycemia in these patients. Finally, the present review concludes that PA should be recommended for the management of patients with

T1DM due to its significant health benefits and protective effects against associated health risks. It also provides suggestions for the future direction of research in this field.

Key Words: Type 1 diabetes mellitus; Physical activity; Health benefit; Glycemic control; Exercise

Core Tip: Diabetes mellitus (DM) is a group of chronic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The impairment of beta-cell function is an ancient feature of disease pathogenesis, while a significant reduction in beta-cell mass is closely associated with clinical manifestations in type 1 DM and type 2 DM. Physical activity (PA) is good for almost every individual. PA is a significant mediator of glycemic control and prevents pathologies related to increased postprandial glucose. Its significant role in the prevention and management of noncommunicable diseases is extensively understood. PA is widely known to be an effective approach for the prevention and management of numerous chronic diseases.

Citation: Wake AD. Protective effects of physical activity against health risks associated with type 1 diabetes: "Health benefits outweigh the risks". *World J Diabetes* 2022; 13(3): 161-184
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/161.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.161

INTRODUCTION

Diabetes mellitus (DM) is a group of chronic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both[1]. The loss of beta-cells (β-cells) is a determinant factor for the development of type 1 DM (T1DM)[2]. In T1DM and type 2 DM (T2DM), the impairment of β-cell function is an early feature of disease pathogenesis, while a significant reduction in β-cell mass is closely associated with clinical manifestations[3]. Reduced functional β-cell mass is the hallmark of both T1DM and T2DM and this triggers absolute or relative insulin insufficiency in both circumstances [4]. T1DM is characterized by an immune-mediated depletion of β-cells that results in a lifelong dependence on exogenous insulin[5-9]. Despite all the efforts made to identify efficient therapeutic methods for T1DM, insulin is the only effective treatment[2].

However, even modest levels of β-cell activity were associated with a reduction in the incidence of retinopathy and nephropathy in T1DM individuals[10]. Even though the factors that predict the occurrence and rapidity of the decrease in β-cell function are still largely unknown, evidence has identified islet cell autoantibodies as predictors. Historical as well as recent clinical experience has underlined the significance of residual insulin production for glycemic control and to avoid end-organ complications[11]. T1DM can arise at any age while a peak in incidence is seen around puberty[12]. Overweight and obesity are highly prevalent among young people and adults with T1DM which is (25%–35%) and (37% to nearly 80%), respectively. Obesity raises the risk of developing T1DM and may lead to an earlier age at diagnosis. Also, obesity may raise the risk of macrovascular disease, retinopathy, and metabolic syndrome among these patients[13]. The chronic hyperglycemia of diabetes is linked to long-term damage, dysfunction, and failure of different organs, particularly the eyes, kidneys, nerves, heart, and blood vessels[1].

Generally, diabetes-related complications can be divided into macrovascular and microvascular complications. Stroke, coronary artery disease, and peripheral arterial disease are included under macrovascular complications and microvascular complications comprise, retinopathy, diabetic nephropathy, and neuropathy[14]. In addition, T1DM is linked with premature cardiovascular disease (CVD)[15]. In T1DM individuals, the absolute and relative risks of CVD remain very high[16]. When women with T1DM are compared to men with T1DM, women have nearly 40% more excess risk of all-cause mortality and twice the excess risk of fatal and nonfatal vascular events[17]. The age at onset and the duration of T1DM seem to be significant determinants of survival and all cardiovascular outcomes. Early onset is associated with up to a 30-fold augmented risk of serious cardiovascular outcomes, with risk levels being 90-fold greater for women with early-onset diabetes, and who die approximately 18 years earlier than nondiabetics[18]. Vascular complications are a significant cause of morbidity and mortality in individuals with T1DM and T2DM[19].

The magnitude of DM has increased in recent decades[20], where the number of cases and the proportion of the disease have been gradually increasing over the past few decades[21]. The incidence of childhood T1DM is significantly increasing globally[22]. It is the epidemic of the century and without effective diagnostic approaches at an early stage, diabetes will continue to increase[23]. The global

estimates for the prevalence of diabetes for 2015 and 2040 were 8.8% and 10.4%, respectively[24]. Whereas it was 8.8% and 9.9% of the world population in 2017 and by 2045, respectively[25].

In recent decades, a significant increase in the proportion of DM has been evidenced in nearly all regions of the world. The increase in the number of subjects with the disease is possibly due to a change in the disease profile in numerous populations around the world and this is primarily because of a larger incidence of diabetes-related complications such as kidney failure and peripheral arterial disease [26]. It is a significant public health problem and is one of the four ranked noncommunicable diseases targeted for action by world leaders[21]. T1DM is associated with an increased risk of CVDs and all-cause mortality in insulin-treated patients with diabetes while the connection between hypoglycemia and cardiovascular consequences and mortality exists over a long period[27]. Whereas, more than the general population, elderly individuals with diabetes have higher all-cause mortality rates[28]. The excess mortality observed in T1DM is almost totally associated with diabetes and its related comorbidities[29].

The increasing disease burden of DM globally is a major public health priority, placing a fluctuating need on the patients, their careers, health systems, and society[30]. Diabetes is one of the leading and rising causes of hospital admission and disability due to other diseases[31]. This high magnitude of the disease has a greater social and financial burden[24]. It imposes a rising economic burden on national health care systems globally[32]. The global costs of DM and its significance are huge and will markedly rise by 2030[33]. Even though the present data found an increase in the magnitude of diabetes, the recent understanding of the international burden of and variation in the disease linked with complications is poor worldwide[26].

Diabetes is a chronic illness that requires ongoing medical care and patient self-management to prevent complications. Diabetes care is complex, requires numerous issues to be addressed, and it is more than glycemic control[34]. T1DM is a chronic disease with severe complications due to its mismanagement. The health professionals should be equipped with suitable evidence based on multiple management approaches to those individuals to support patient-centered care and improve their capacity for problem-solving and self-management[35]. Maintaining the long-term integration of lifestyle changes and medical management is crucial to accomplish good metabolic control in diabetes subjects[14,36].

Self-management participation could lead to clinically associated progress in the behavior and clinical parameters[37] and those individuals who participate in self-management can be considered volunteers in the majority of cases where they have either wanted an intervention or decided to take part[38]. Physical activity (PA) and nutrition are significant components of a healthy lifestyle and treatment of diabetes[39]. The benefit of exercise in T1DM remains a significant component of its treatment[40]. Therefore, the adoption and maintenance of PA are crucial for the management of glycemia and the entire health of individuals with diabetes and prediabetes[41]. Exercise is a cornerstone in the lifestyle of nearly all individuals with T1DM[42]. As the patients may be more agreeable to lifestyle changes, exercise should be encouraged from diagnosis. In addition, to improve patient confidence in managing their diabetes with exercise, standard advice on exercise and diabetes needs to be made available to health professionals and subjects with diabetes[43].

With regard to PA, even though, the term PA and exercise are not synonymous they are often used interchangeably[44,45]. However, the term "PA" should not be mistaken with "exercise", as exercise is a subgroup of PA[46]. Due to this, it is recommended that they should not be used interchangeably[47]. PA can be defined as any bodily movement formed by the skeletal muscles that result in energy expenditure above resting levels[46,48-50]. While exercise is defined as a planned, structured PA typically performed with the intent of improving health and/or fitness[46,48]. The term PA is broadly comprised of exercise and sport, and PA is performed as a part of daily living, occupation, leisure, and active sport[46,48]. Exercise can be classified as aerobic and resistance exercise[51]. Aerobic exercise involves the repeated and continuous movement of huge muscle groups[52]. Anaerobic exercise comprises activities such as walking, cycling, jogging, and swimming. Resistance exercise includes activities such as free weights, weight machines, bodyweight, or elastic resistance bands[51].

METHODS

Research questions

What are the protective effects of physical activity against the health risks associated with T1DM?

Study setting

The present review article includes all studies conducted in various countries globally.

Search strategies

The present review article was carried out using a comprehensive literature search. The search was performed using the following electronic databases: “Cochrane Library”, Web of Science, PubMed, HINARI, EMBASE, Google for grey literature, Scopus, African journals Online, and Google Scholar. The

search was conducted using the following search terms; "diabetes mellitus", "type 1 diabetes", "T1DM", "complications", "insulin-dependent diabetes mellitus", "IDDM", "physical activity", "exercise", "Glycemic Control", "Physical Fitness", "Blood Lipids Profile", "Endothelial Function", "Insulin Resistance", "Insulin Sensitivity", "Insulin Requirement", "Cardiovascular Diseases", "Blood Pressure", "Well-being", "Body's Defense Systems", "Mortality", "barriers", "factors", "strategy", and "hypoglycemia". The Boolean operators; "AND" and "OR" were used to integrate them during the search.

Eligibility criteria

The inclusion criteria for the present review were; Articles on this topic globally, published in the English language, quality articles, and those with outcome variables well defined and measured, and articles published up to June 21, 2021. The exclusion criteria for the present review article were: articles of poor quality and articles in which the outcome variable was not clearly defined and measured.

THE HEALTH BENEFITS OF PHYSICAL ACTIVITY FOR T1DM PATIENTS

Overall, PA is good for almost every individual[52]. It provides numerous health benefits mainly for obese individuals[53]. Even among healthy individuals, daily PA is a significant mediator of glycemic control and enhances the prevention of pathologies related to increased postprandial glucose[54]. Its significant role in the prevention and management of noncommunicable diseases is extensively understood[55]. Exercise is largely known as an effective approach for the prevention and management of many chronic diseases[56]. It is essential in the primary and secondary prevention of chronic diseases such as CVD, diabetes, cancer, hypertension, obesity, depression and osteoporosis, and premature death [57]. The effects of exercise also include the management of many metabolic syndromes as well as improved mood and quality of life[58].

Exercise training leads to improved body composition, cardiovascular, and metabolic outcomes in subjects with metabolic syndrome[59]. Moreover, PA helps to decrease all causes of morbidity and improves the quality of life in people of all age groups[60,61]. It also benefits principally older adults by protecting against and ameliorating several diseases, the achievement and maintenance of a healthy body weight, improved mental health and well-being, and musculoskeletal health. The amelioration of disease risk factors, the achievement of peak bone mass, and maintenance of healthy body weight were the benefits of PA in children[62].

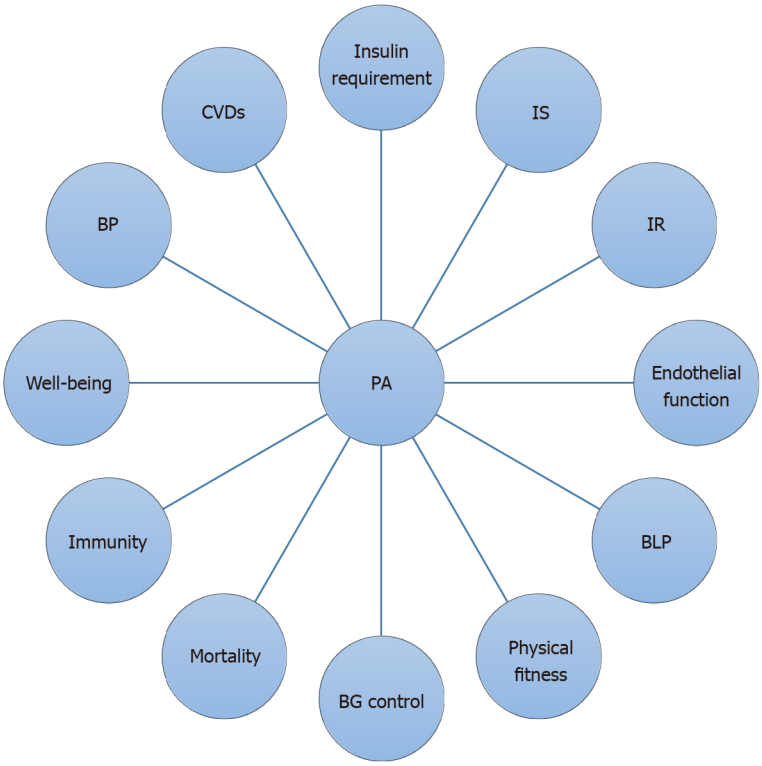
Activity may play a protective role due to the consistent relationship between historical PA and the development of complications in insulin-dependent DM (IDDM)[63]. Exercise also has many significant health merits in both T1DM and T2DM subjects[64] and many exercises are supportive in these subjects [65]. The findings from randomized trials support the role of resistance training as an adjunctive mode of management in T1DM patients[66]. Both aerobic and resistance exercises are excellent for patients with T1DM[67]. In addition, regular moderate to vigorous PA was linked with many health benefits in adolescents with T1DM[68]. Exercise decreases the rate of diabetes-related complications in T1DM subjects[69].

Vigorous-intensity PA has a role in metabolic control in T1DM patients[70]. Consistent regular PA can improve metabolic control in these patients[71] and is significant for best physical and psychological development during childhood, and it improved glycemic control, cardiovascular function, blood lipid profiles, and psychological well-being[72]. Consistent PA has a beneficial effect on glycemic control, diabetes-related comorbidities, and cardiovascular risk factors without the risk of adverse events[73]. A summary of the effects of PA on many health outcomes such as blood glucose (BG) control, physical fitness, endothelial function, insulin sensitivity, well-being, body defense system, blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality is shown in Figure 1.

GLYCEMIC CONTROL

Discrepancies have been observed in the literature regarding the role of PA in T1DM glycemic control. For instance, in T1DM female individuals, daily physical training for several months did not improve glycemic control[74]. In addition, Yki-Jarvinen *et al*[75] demonstrated that a controlled physical training program in pump-treated T1DM subjects did not change an already near-normal glycemic control. Furthermore, Zinman *et al*[76] found that, although plasma glucose declines acutely with exercise, an augmented caloric intake on exercising days obviates the long-term effect of training on glucose control. Similarly, glycemic control did not significantly improve in pregnant women with T1DM during postprandial walking exercise[77]. Moreover, glycemic control was not found to be associated with long-term PA in T1DM subjects and PA did not negatively affect long-term glycemic control[78].

Several studies have found that PA improved glycemic control in individuals with T1DM[69,71,79-113]. Regular PA can lead to decreased BG level among these patients, it is safe and does not result in



DOI: 10.4239/wjd.v13.i3.161

Figure 1 The health benefits of physical activity in type 1 diabetes patients. PA: Physical activity; BG: Blood glucose; BLP: Blood lipid profile; IR: Insulin resistance; IS: Insulin sensitivity; CVDs: Cardiovascular diseases; BP: Blood pressure.

more hypoglycemic episodes[91]. A systematic review showed that PA had a positive impact on glycemic control in children and adolescents with T1DM[97]. Similarly, regular PA enhances BG control in children with T1DM[98]. Prolonged moderate aerobic exercise leads to a consistent decrease in plasma glucose but frequent hypoglycemia can occur when pre-exercise glucose concentrations are < 120 mg/dL in young people with T1DM[100]. Increased leisure-time PA (LTPA) between the ages of 50 and 70 years in the absence of active intervention was also found to be associated with improved glucose in men[101]. Campaign *et al*[71] demonstrated that regular high-intensity PA can improve metabolic control in young children with IDDM. In addition, combined exercise training (endurance training and resistance training) improves glycemic control to a better extent than endurance or resistance training alone, under moderate-intensive training situations with equal training durations [82]. Supervised strength training in T1DM male patients was associated with significant changes in glycemic control[93]. Marrone *et al*[95] found that free-play PA has a crucial role in helping to maintain BG levels in children with T1DM. Furthermore, anaerobic circuit training was found to improve glucose regulation in adolescents with IDDM[96]. Regular participation in moderate to intense PA or sports improves metabolic control in T1DM subjects[103]. High-intensity training (HIT)[105] and resistance training[106] improve plasma glucose in T1DM patients. Generally, an enhanced skeletal muscle, by either an intrinsic mechanism or PA, provides better advantages and benefits in facilitating glucose regulation[86] as peripheral glucose utilization rises during exercise, despite a reduction in circulating insulin levels[85]. During PA, muscle glucose uptake also rises and can reach values that are 30-50 times greater than at rest[87].

PA decreased glycosylated hemoglobin (HbA1c) in T1DM patients[81,89,90,111-113]. This effect is acceptable since the HbA1c level is increased following PA cessation[89]. This shows that the reduction of HbA1c level is a major sign of glycemic control. This is because the amount of glucose that combines with HbA1c is directly proportional to the total amount of glucose within a system. This means, if the BG levels have been high in current weeks, the HbA1c level will also increase. This could be evidence of PA reducing BG level, and was proved by the decrease in this biomarker of glycemic control.

PHYSICAL FITNESS

Physical fitness is defined as a set of attributes that are either health- or skill-related and the extent to which individuals have these attributes can be measured with specific tests[50]. Evidence shows that patients with T1DM have reduced physical fitness[114]. Furthermore, children with T1DM presenting

with poor glycemic control had lower aerobic fitness compared to those with good glycemic control [115]. In addition, lower cardiorespiratory fitness in children with T1DM is associated with poor glycemic control[116].

However, numerous studies have shown that PA improves physical fitness in individuals with T1DM [92,93,96,97,117,118]. Supervised strength training in male patients was associated with augmented strength[93]. Also, exercise training among adolescents with T1DM leads to improved physical fitness [92]. Even, a training program of 1 h per week for 3 mo was found to improve physical fitness[117]. Anaerobic circuit training improved muscle strength in adolescents with IDDM[96]. Furthermore, a systematic review showed that PA improved physical fitness in children and adolescents with T1DM [97]. Moreover, a randomized trial demonstrated that combined exercise training appeared to improve physical fitness in adolescents with T1DM[118]. Regular PA also improved cardiovascular fitness[71,98, 102,107] and increased lean mass in these patients[98].

BLOOD LIPIDS PROFILE

A study found that youths with T1DM have abnormal lipid levels and atherogenic changes in lipoprotein composition, even after a relatively short disease duration, and glycemic control is a significant mediator of these abnormalities[119]. In normal-weight T1DM youths, mainly females had more atherogenic low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) distributions which are associated with lower insulin sensitivity[120]. Dyslipidemia is significantly more frequent in children and adolescents with T1DM compared to non-diabetic peers whilst high LDL-C and low HDL-C were the most frequent type of dyslipidemia in the dyslipidemic group[121]. Similarly, dyslipidemia is frequently found in T1DM and appears to be associated with glycemic control. It is a major risk factor for coronary heart disease, and one of the most significant and frequent complications with a high premature mortality and morbidity rate[122]. In addition, apolipoprotein B is consistently associated with an increased risk of mortality in T1DM due to all causes as well as in cardiac disease and ischemic heart disease[123].

Several studies have proved that PA decreases the blood lipids profile in individuals with T1DM[69, 74,80,93,98,101-103,110,111,124-128]. Laaksonen *et al*[125] found that endurance training improved the lipid profile in physically active T1DM men. Anaerobic circuit training also improved the lipid profile in adolescents with IDDM[96]. Similarly, regular PA improved the blood lipid profile and reduced body adiposity in children with T1DM[98]. In addition, there is a linear dose-response relationship between augmented PA and loss of abdominal fat in T1DM[91]. Regular exercise had beneficial effects on body fat content and the lipoprotein profile in subjects with T1DM by decreasing high plasma lipoprotein(a) concentrations[128]. Similarly, PA improved the lipoprotein profile in T1DM patients[102]. Increased PA in children with T1DM is related to a lower lipoprotein level[80]. Daily training for a number of months in T1DM females had a significant effect on the HDL3-C subfraction but led to minor changes in serum lipoprotein profiles[74]. Austin *et al*[127] demonstrated that the state of physical fitness was significantly correlated to lipid levels and lipoprotein(a) in adolescents with IDDM, where higher physical fitness levels decreased lipid levels. Total cholesterol (TC) levels significantly declined after an exercise intervention[124]. Consistent supervised strength training among male patients with T1DM was associated with a reduced TC level[93]. A systematic review also showed that PA reduced the TC level in children and adolescents with T1DM[97]. In addition, physical training in IDDM leads to reduced TC levels[126]. Yki-Jarvinen *et al*[75] found that a controlled physical training program in pump-treated diabetic patients increased the HDL-C to TC ratio in T1DM subjects. Postprandial walking exercise in pregnant women with T1DM was associated with significantly lower fasting plasma triglyceride levels in an intensive perinatal diabetes program[77]. Regular moderate to intense PA or sports participation improved the lipid profile in T1DM subjects[103]. Also, augmented LTPA between the ages of 50 and 70 years in the absence of active intervention was associated with improved lipid metabolism in men[101].

ENDOTHELIAL FUNCTION

Evidence shows that endothelial dysfunction is common in adolescents with T1DM[129] and it is a predictor of CVDs in these patients[130]. Studies have found that PA improves endothelial function in individuals with T1DM[110,131,132]. Anaerobic exercise training can improve endothelial function in different vascular beds in individuals with long-standing T1DM who are at substantial risk of diabetic angiopathy[131]. However, regular exercise training involving the lower extremities did not improve endothelial function in the micro- and macro-circulation of the non-exercised upper extremity in T1DM individuals[132].

INSULIN RESISTANCE

T1DM subjects are insulin resistant compared with nondiabetic subjects[133], and insulin resistance in the liver and skeletal muscle was found to be a significant characteristic in T1DM[134,135]. Youths with T1DM have adipose, hepatic, and peripheral insulin resistance[136]. This insulin resistance is an independent risk factor for the development of macro-and microvascular complications and may also contribute to the development of the disease[137]. Insulin resistance could also impact the length of the honeymoon period, diabetic control and patterns of growth during puberty, insulin requirements and BG control at any time, the birth weight of infants born to diabetic mothers, lipid metabolism, hypertension, rates of progression to insulin dependence and eventually contribute to excess mortality [138].

Insulin resistance is linked with a greater atherogenic lipoprotein cholesterol distribution in all men and women with T1DM[139]. Gender differences in insulin resistance-associated fat distribution may clarify why T1DM increases coronary calcification in women more than in men[140]. Greater insulin resistance was found in a group of premenopausal women with T1DM compared with nondiabetic subjects which was not related to abdominal adiposity, lipids, or androgens[141].

Studies have shown that PA improves insulin resistance in patients with T1DM[142,143]. For instance, aerobic exercise decreases waist circumference which is related to a tendency for raised HDL-C levels, and this may indicate a decrease in visceral fat with an improvement in insulin resistance[143]. This finding is also supported by the trial conducted by Dotzert *et al*[144] where aerobic exercise training was found to improve insulin resistance in insulin-resistant T1DM rats. The capacity of exercise to increase insulin-stimulated glucose uptake *in vivo* was decreased in subjects with insulin-resistant T1DM compared with normal individuals, and this could have been due to either separate or common defects in exercise- and insulin-stimulated pathways[142]. In β-cell transplanted recipients, endurance training may be helpful and preventive by counteracting graft dysfunction, alleviating the side effects of immunosuppressive drugs, and conserving insulin independence after islet transplantation[145].

INSULIN SENSITIVITY

T1DM adolescents have significantly reduced insulin sensitivity compared with nondiabetic adolescents [146]. Several studies have verified that PA improves insulin sensitivity in patients with T1DM[39,75,83,84,92,98,101,126,147,148]. Augmented LTPA levels were associated with raised insulin sensitivity[84,101]. Regular PA enhanced insulin sensitivity in children with T1DM[98]. Also, a controlled physical training program in pump-treated T1DM subjects improved body sensitivity to insulin[75]. Exercise training in adolescents with T1DM can lead to improved insulin sensitivity[92]. Physical training in IDDM also leads to raised peripheral insulin sensitivity[126]. Regular moderate-intensity PA can improve insulin sensitivity in T1DM individuals[39]. The findings from the trial, where exercise led to improved insulin sensitivity and responsiveness by different mechanisms in rats[149] also supports these studies.

INSULIN REQUIREMENT

PA also decreases insulin requirements in subjects with T1DM[75,84,128]. A controlled physical training program in pump-treated T1DM subjects decreased insulin requirements[75]. In addition, male patients with T1DM appeared to use less insulin when they were physically active[84]. Furthermore, regular exercise[128] and combined exercise training (aerobic and resistance) appears to lower daily insulin requirements in patients with T1DM[118]. Similarly, a study showed that exercise can improve insulin requirements in T1DM rats[150].

CARDIOVASCULAR DISEASES

Normal weight adolescents with T1DM have impaired autonomic function and augmented energy expenditure and fat oxidation compared to individuals without diabetes who have similar levels of fitness and PA[151]. T1DM adolescents had significantly reduced peak oxygen consumption (VO2peak), and peak work rate compared with nondiabetics. They also had decreased vascular reactivity, diastolic dysfunction, and left ventricular hypertrophy[146]. Maximal workload and oxygen uptake were markedly diminished in chronically hyperglycemic IDDM subjects and physiologically significant cardiopulmonary dysfunction developed in asymptomatic patients with long-standing disease[152]. Insulin resistance in T1DM may contribute to the augmented CVD burden[120]. In T1DM, heart rate variability and arterial wall stiffness are linked to each other where the autonomic nervous system could be a connection between diabetes and vascular disease[153]. In African Americans with T1DM, high

plasma interferon-inducible protein 10 was found to be an independent predictor of incident CVD[154].

Studies have confirmed that PA decreases the risk of CVDs in T1DM patients[39,80,91,104,110,112,124,127,128,143,155-159]. Increased PA in children with T1DM has a beneficial effect on the CV risk profile[80]. There is a linear dose-response relationship between augmented PA and a decrease in lipid-related CV risk factors, with a preferential rise in the HDL3-C subfraction in patients with T1DM[91]. Exercise also improves diabetic complications such as subclinical autonomic neuropathy and CVD risk in children with T1DM[124]. Higher physical fitness levels due to exercise decrease lipid levels and this, in turn, may reduce the risk of CVD[127]. In addition, regular exercise may decrease CV risk in T1DM patients by decreasing high plasma lipoprotein Lp(a) concentrations[128]. Aerobic exercise decreases waist circumference which is related to a tendency for raised HDL-C levels, and this may indicate a decrease in visceral fat with an improvement in insulin resistance which could have an influence on reducing CV risk in these patients[143]. An inverse association was found between PA and the incidence of CVD in women with T1DM[155]. Also, high frequency and high-intensity exercise may decrease the risk of CVD in individuals with T1DM[156]. Regular moderate-intensity PA can decrease the risk of CVD in T1DM individuals[39]. Furthermore, PA has the potential to delay CVD in T1DM as it reduces the risk of CVD[104,112,157]. Integrated with diet, it can also influence lipid-related CV risk factors independent of changes in insulin treatment[158]. T1DM subjects who are physically more active have a lower overall risk of CV events than their sedentary counterparts[159]. Moreover, the study showed that aerobic circuit training was found to improve cardiorespiratory endurance[96] and regular PA improved vascular health in those subjects[160].

BLOOD PRESSURE

PA also improved blood pressure (BP) in patients with T1DM[80,91,102,124,128]. A study showed that regular exercise was found to have beneficial effects on BP in these patients[128]. Increased PA in children with T1DM leads to improved diastolic BP (DBP)[80]. A linear dose-response relationship between increased regular PA and decreased BP in patients with T1DM was also found[91]. DBP and heart failure were significantly correlated with lower TC levels which were lowered by performing exercise[124]. In addition, adolescents with T1DM have been associated with reduced stroke volume during exercise[161]. T1DM adolescent girls showed decreased sympathetic activity, although this was possibly compensated by higher adrenomedullary responsiveness or sensitivity, and did not affect their heart rate adaptation to exercise[162].

WELL-BEING

Studies have also found that PA improves the well-being of individuals with T1DM[39,69,97,98,118]. A systematic review showed that PA improved the well-being and psychological health of children and adolescents with T1DM[97]. Regular PA enhanced psychosocial well-being in children with T1DM[98]. A randomized trial demonstrated that combined exercise training (aerobic and resistance) appeared to lead to better well-being in adolescents with T1DM[118]. Also, regular moderate-intensity PA can improve the psychological well-being of these individuals[39]. Furthermore, Brazeau *et al*[163] demonstrated an association between greater PA and a better body mass index, body composition, and more favorable health status in individuals with T1DM similar to individuals without diabetes.

BODY'S DEFENSE SYSTEMS

T1DM individuals have higher levels of free radicals and may, as a result, be at augmented risk of developing complications related to T1DM[164]. PA has been found to protect against protein denaturation[165]. Aerobic training improves oxidative stress in individuals with diabetes[110]. Acute exercise is an immune system adjuvant that improves defense activity and metabolic health. In addition, there is a clear inverse relationship between moderate exercise training and illness risk[166]. Farinha *et al*[9] demonstrated that exercise training improves the body's defense systems and metabolic health in T1DM patients and induces numerous benefits by decreasing inflammation and improving antioxidant defenses.

MORTALITY

Mortality rates in the past decade continue to be much larger in individuals with T1DM than in those without diabetes despite advances in inpatient care[167]. The risk of death rises with less favorable

glycemic status and impaired carbohydrate metabolism contributes to mortality from any cause[168]. The mortality rate due to ischemic cardiac disease is greater in T1DM patients compared with the general population[169]. In these patients, the presence of metabolic syndrome is frequent, and it is linked with an augmented incidence of chronic complications and mortality[170]. Macrovascular and microvascular disease are the main causes of mortality in T1DM[130]. In addition, physical inactivity has been found to contribute to a substantial number of deaths in those with DM[171]. A significantly increased mortality risk due to diabetes was associated with decreased health-related quality of life in subjects who reported no LTPA[172].

Many studies have shown that PA decreases mortality in subjects with T1DM[93,143,155,172-175]. Supervised strength training in men with T1DM was related with no morbidity[93]. An inverse association was found between PA and all-cause mortality in both genders with T1DM[155]. Participating in LTPA may be linked with improved survival in patients with diabetes[172]. Furthermore, exercise is associated with a lower risk of premature all-cause mortality such as CVD and chronic kidney disease in patients with T1DM[173], and PA has been found to decrease CV mortality in these subjects[143,174]. Moreover, PA offers a beneficial effect in terms of long life in IDDM patients [175]. In African Americans with T1DM, low plasma stromal-derived factor-1 was found to be an independent predictor of mortality[154].

THE BARRIERS TO PHYSICAL ACTIVITY PRACTICE IN T1DM PATIENTS

Even though several literature reports have supported the utilization of PA for individuals with T1DM, most of the patients did not engage in regular exercise due to various obstacles. For instance, the fear of exercise-induced hypoglycemia is the strongest barrier to regular PA in adults with T1DM[39,176-180]. Although technological advances have permitted exercisers with diabetes to progress toward more successful management of their BG levels during various types of PA, technology is still far from fully avoiding the fear of hypoglycemia in T1DM subjects[176]. Glucoregulatory failure may cause hypoglycemia in IDDM individuals during and after exercise. This could be due to hypoglycemic episodes which blunt the glucoregulatory response to subsequent exercise while exercise blunts the glucoregulatory response to subsequent insulin excess[181]. Even though regular PA was found to improve glycemic control, its frequency is a major factor affecting the control of glycemia without raising the risk of severe hypoglycemia in pediatric patients with T1DM[94]. This may be supported by a study, where low levels of LTPA were associated with poor glycemic control in T1DM women[84].

Adolescents with T1DM who participate in moderate-intensity exercise in the afternoon have augmented glucose needs at the time of and shortly after the completion of exercise. In addition, the reduced counter-regulatory responses to hypoglycemia post-exercise may lead to a higher risk of hypoglycemia overnight[182]. Antecedent hypoglycemia induces acute counter-regulatory failure both during subsequent hypoglycemia and moderate exercise in T1DM. This acute state of counter-regulatory impairment may be one cause of exercise-associated hypoglycemia in these individuals[183]. Anaerobic exercise usually causes BG concentration to reduce quickly, whereas anaerobic exercise may cause it to increase, making glycemic control challenging for patients with T1DM[184]. Prolonged exercise could lead to hypoglycemia even in normal male individuals[185]. Even though the risk of exercise-induced hypoglycemia is a great challenge for these patients, the glycemic response to exercise depends upon several factors concerning the patient him/herself such as therapy, glycemic control, training level, and the characteristics of the exercise performed[186].

Also, evidence shows that there are sex-related differences in exercise responses that might affect BG levels during exercise in patients with T1D[187,188]. Marked sexual dimorphism occurs in the pattern of counter-regulatory responses to moderate, prolonged euglycemic exercise in subjects with T1DM. Despite decreased plasma levels of epinephrine, norepinephrine, and growth hormone, T1DM women have a higher lipolytic response, which probably reflects greater tissue sensitivity to one of these hormones during exercise[188]. When compared with men, women with T1DM are more resistant to the blunting effects of antecedent hypoglycemia on neuroendocrine and metabolic responses to subsequent moderate exercise[189].

A study showed that patients with T1DM have a variable glycemic response to prolonged aerobic exercise, and this variability is partially explained by their pre-exercise BG levels[190]. High-intensity interval training (HIIT) has been found to improve anaerobic capacity without a detrimental decrease in BG in these patients[191]. However, another study showed that HIIT in fasting individuals with T1D produces a large and consistent hyperglycemic response instantly post-exercise[192]. Also, Fahey *et al* [193] demonstrated that a sprint as short as 10 s can raise plasma glucose levels in nondiabetic and T1DM subjects, with this increase resulting from a transient decrease in glucose rate of disappearance (Rd) rather than from a disproportionate rise in glucose rate of appearance (Ra) relative to glucose Rd as reported with intense aerobic exercise. Furthermore, other identified barriers were lack of time and work-related factors, access to facilities, lack of motivation, embarrassment and body image, weather, and having low levels of knowledge about managing diabetes and its complications in relation to exercise as the main barriers to perform exercise[194].

THE STRATEGY TO CONTROL PHYSICAL ACTIVITY INDUCED HYPOGLYCEMIA IN T1DM

The effective management of T1DM desires a multidisciplinary combined method to develop individualized programs, attention to all factors that may influence the result, and the expectations of those with TIDM should be paramount in the strategy adopted by the diabetes care team[195]. It is essential to know that both hypoglycemia and hyperglycemia can arise during exercise; however, strategies are available to deal with these challenges[64]. In T1DM, due to the potential risk of hypoglycemia, the patients must be carefully educated about the consequences of PA on their BG levels and the modifications of diet and insulin therapy before starting exercise sessions[196].

It is supportive for subjects to monitor their BG levels before, during, and after exercise, to avoid T1DM complications and to identify when changes in insulin or food intake are essential. In particular, individuals who experience late or nocturnal hypoglycemia should have a snack after exercise and/or before going to sleep[197]. It is suggested that the personalized exercise carbohydrate requirement estimation system can be used for the management of exercise-related glycemic imbalances in T1DM [198]. In individuals with T1DM being treated with intensive insulin therapy containing the basal-bolus (NPH-human regular) insulin regimen, walking after meals improves glycemic control[199]. Also, performing resistance exercise before aerobic exercise improves glycemic stability throughout exercise and decreases the duration and severity of post-exercise hypoglycemia in subjects with T1DM[200]. Performing a morning resistance exercise session after an overnight fast and omission of pre-exercise rapid-acting insulin does not induce acute post-exercise hypoglycemia or increase the marker of muscle damage in T1DM patients[201]. In addition, morning exercise reduces the risk of late-onset hypoglycemia compared with afternoon exercise and improves BG control the following day[109]. Eating low glycemic index food with a decreased rapid-acting insulin dose following evening exercise avoids postprandial hyperglycemia and inflammation and provides hypoglycemia protection for nearly 8 h post-exercise[202]. Ingested carbohydrates before moderate-intensity exercise with added repeated sprints is not significantly detrimental to glycemic management in overnight fasted people with T1DM under basal insulin conditions[203]. Also, a qualitative study among athletes with T1DM showed that peer mentoring and mobile apps could potentially support the management of glycemic control in athletes[204]. In athletes with T1DM, while the reductions in glucose level during continuous moderate-intensity exercise and combined (continuous moderate-intensity and intermittent high-intensity) exercise are analogous, the latter form of exercise protects against nocturnal hypoglycemia which indicates that continuous moderate-intensity exercise is linked with a raised risk of nocturnal hypoglycemia in these patients[205].

Furthermore, a larger insulin basal rate decrease and supplemental carbohydrates during exercise may be essential to avoid hypoglycemia[206]. In addition, a combination of ideal glycemic control, empirical adjustments of insulin administration at the time of exercise, and ingestion of carbohydrate supplements tailored to the type, intensity, and duration of an exercise also help to prevent hypoglycemia[207]. Exercise has a role in insulin pump therapy and improves metabolic control in patients with T1DM[208]. A reduction in the basal rate during fasting exercise in continuous subcutaneous insulin infusion-treated individuals seems to be a reasonable step in the maintenance of near-normoglycemia in individuals in whom this occurs[209].

However, reducing the basal insulin infusion rate by 80% up to 40 min pre-exercise onset was found to be insufficient to decrease exercise-induced hypoglycemia[210]. In children, even discontinuing basal insulin during exercise is an effective approach for reducing hypoglycemia in children with T1DM, but the risk of hyperglycemia is increased[211]. Another approach is short-time hypoxia together with graded exercise, which increases cardiorespiratory adaptation to exercise and permits more effective control of glucose homeostasis in T1DM[88]. Combining exercise with hypoxia may permit more effective short-term glycemic control in these patients[212]. Besides, home-HIT appears to provide a strategy to decrease the fear of hypoglycemia, while simultaneously eliminating other identified barriers in individuals with T1DM from performing exercise such as being time-efficient, no travel time or costs related to gym memberships, and providing them with the chance to exercise in their chosen environment, decreasing embarrassment experienced by some when exercising in public[213].

Technology such as continuous glucose monitoring (CGM) is a strategy to control hypoglycemia during exercise in T1DM, and it allows individuals to see the trends in glycemic fluctuations when exercising and in the subsequent night to deal pre-emptively with hypoglycemic risks and treat hypoglycemic episodes in a timely way[214]. Using CGM during exercise may avoid exercise-induced hypoglycemia, but usual BG control should be carried out during intensive exercise[215]. High-intensity exercise leads to delayed nocturnal hypoglycemia and CGM is a useful approach in T1DM subjects who undergo an exercise program[216].

Using CGM trends and carbohydrate intake based on standard exercise carbohydrate intake guidelines to facilitate exercise in children with T1DM is effective as both were found to minimize hypoglycemia and maintained euglycemia during exercise in young children with T1DM[217]. In addition to this, real-time continuous glucose monitoring (RT-CGM) can be recommended as an extra tool that offers T1DM adolescents a rapid reaction to decrease glycemic variability within a short time [218]. Besides, RT-CGM with a carbohydrate intake algorithm may avoid hypoglycemia and maintain euglycemia during exercise, mainly if the subject consumes carbohydrates when trend arrows alert

them to a drop in glycemia[219].

Furthermore, closed-loop insulin delivery also offers an effective means to decrease the risk of nocturnal hypoglycemia while increasing the percentage of time spent in the target range, irrespective of activity level during mid-afternoon. This could benefit these patients even if it is limited to the overnight period[220]. The hybrid closed-loop systems are another approach that help to avoid hypoglycemia, relying on accurate carbohydrate ratios and carbohydrate counting, and the algorithm that was tested against moderate exercise and an over-reading glucose sensor performed well in terms of hypoglycemia prevention[221]. Moreover, a study demonstrated that the heart rate–enhanced artificial pancreas system improved protection against hypoglycemia during exercise in T1DM[222]. Several studies have shown different strategies that could be integrated with exercise as a means of glycemic control in individuals with T1DM (Table 1)[223-240].

PHYSICAL ACTIVITY RECOMMENDATIONS FOR T1DM PATIENTS

It is essential to balance the risks of insulin-induced hypoglycemia with the risks related to poorly controlled diabetes and poor physical fitness in individuals with T1DM[241]. Considering the risk-benefit ratio, several studies have recommended PA in patients with T1DM[9,71,79,92,97,113,124,158,191,207,214,231,242-248]. Regular PA should be a routine aim in these subjects, for various health and fitness reasons. However, considerable challenges remain for these patients, and their healthcare team, in the management of exercise and sports[244]. Exercise is highly recommended for patients with T1DM as it has several beneficial health effects, with the prevention of long-term cardiovascular complications being dominant[207,214].

With regard to exercise, regular moderate-to-vigorous exercise should become a central part of the management of subjects with T1DM, in the absence of contraindications and accompanied by all desirable educational support for optimal diabetes management[245]. Children with IDDM can be engaged in regular vigorous PA (with minimal risks)[71] and regular exercise[124]. A combined exercise of strength training (ST) and HIIT for at least 2 mo, 3 times per week, will provide many health benefits for T1DM subjects[9].

Another study showed that HIIT sessions reduced glycemia to a greater degree than ST or ST+HIIT sessions over 10 wk in real-life situations. Because of this, T1DM individuals who develop severe exercise-associated hypoglycemia and/or present pre-exercise capillary glucose levels close to 5.5 mmol/L are recommended to carry out ST or HIIT after ST as the preferred option[247]. Resistance exercise was also found to have several benefits and should be recommended as a significant activity for health and well-being in these individuals, although caution with regard to BG levels will always be essential while performing resistance exercise[231]. However, HIIT may be the chosen training approach for some individuals with T1DM as it has been found to improve anaerobic capacity without a detrimental decrease in BG in these patients[191]. For T1DM patients using ultra-long-acting insulin, both aerobic high-intensity interval exercise and moderate continuous exercise can be safely performed [67].

In addition, high-intensity exercise does not raise the risk of early post-exercise hypoglycemia in patients with T1DM[249]. High-intensity bouts linked with high-intensity exercise result in a more rapid and higher increase in endogenous glucose production during exercise than moderate-intensity exercise alone. During early recovery from exercise, glucose use reduces following high-intensity exercise, while leftovers raised after moderate-intensity exercise despite the performance of more whole work[250]. In pediatric patients with T1DM, the frequency of regular PA was the main factor that affected the control of BG without raising the risk of severe hypoglycemia and this is why it is recommended in these patients[94].

Moreover, for subjects with T1DM, the emphasis must be on adjusting the therapeutic regimen to allow safe participation in all forms of PA consistent with an individual's desires and goals. Eventually, all subjects with diabetes should have the chance to benefit from the valuable effects of PA[248]. Daily PA should be recommended in these patients as part of their management[113] and could be used as an adjunct in glycemic control[92]. Diet and PA can influence glycemic control in IDDM independent of changes in insulin treatment[158].

Besides exogenous insulin therapy and CGM, exercise is recommended in adults with T1DM to improve the entire health of individuals[79]. However, to perform regular PA, the patient and those who supervise them should be aware of disease-specific recommendations and contraindications[242]. Evidence recommends that even individuals with ketonemia may engage in intensive physical training, provided this is part of a program including adequate insulin dosage, dietary advice, and close supervision with multiple daily BG measurements[246]. Evidence suggests that it is significant to consider the needs of the wider support network, as well as the child's or adolescent's concerns and preferences, during the development of new or existing strategies and programs to promote PA in children and adolescents with T1DM[251]. Finally, during PA, the parameters such as supervision, duration, frequency of sessions, protocols with mixed PA may positively affect the metabolic outcome of patients with T1DM[97].

Table 1 Summary of studies on exercise intervention in type 1 diabetes mellitus patients to improve glycemic control

Ref.	Year	Intervention	Findings
Sonnenberg *et al*[223]	1990	CSII during exercise	Hypoglycemia could only be avoided when the premeal insulin bolus was decreased by 50% and discontinuation of the basal insulin infusion during exercise
Rabasa-Lhoret *et al*[224]	2001	Premeal insulin dose reductions forpost-prandial exercises	Minimized risk of hypoglycemia during postprandial exercises of different intensities and different durations by a suitable decrease in premeal insulin lispro
Dubé *et al*[225]	2005	Glucose supplement during exercise in subjects using N-lispro	For 60 min of late post-prandial exercise followed by 60 min of recovery, an estimated 40 g of a liquid glucose supplement, ingested 15 min before exercise was good for BG control
Diabetes Research in Children Network (DirecNet) Study Group *et al*[211]	2006	Suspension of basal insulin during exercise	Basal insulin suspension decreases hypoglycemia from 43% to 16% in individuals, but hyperglycemia 45 min after exercise was more frequent
Bussau *et al*[226]	2006	Ten-second sprint after moderate-intensity exercise	This avoided early post-moderate intensity exercise hypoglycemia
Bussau *et al*[227]	2007	Ten-second sprint before moderate-intensity exercise	Prevented hypoglycemia during early recovery from moderate-intensity exercise
West *et al*[228]	2010	Reductions in pre-exercise rapid-acting insulin by 75%, 50%, or 25%	A 75% reduction in pre-exercise insulin resulted in the greatest preservation of BG, and a decreased dietary intake, for 24 h after running
Taplin *et al*[229]	2010	20% reduction of basal rate overnight	Was safe and effective in preventing nocturnal hypoglycemia
		2.5 mg bedtime dose of oral terbutaline	Effective at avoiding hypoglycemia, but linked with hyperglycemia
Riddell *et al*[219]	2011	RT-CGM and carbohydrate intake algorithm (8-20 g), depending on the concentration of glucose at the time of RT-CGM alert and rates of change in glycemia	The coupled carbohydrate intake algorithm with RT-CGM avoided hypoglycemia and maintained euglycemia during exercise
Garg *et al*[230]	2012	An automatic suspension of insulin delivery when BG ≤ 70 mg/dL during or after exercise	This significantly decreased the duration and severity of induced hypoglycemia without causing rebound hyperglycemia
Yardley *et al*[200]	2012	Resistance exercise before aerobic exercise	Performing resistance first improved glycemic stability throughout the exercise and decreased the duration and severity of post-exercise hypoglycemia
Yardley *et al*[231]	2013	Resistance *vs* aerobic exercise	Resistance caused a less initial decline in BG but prolonged decreases in post-exercise glycemia than aerobic exercise
Campbell *et al*[232]	2013	Pre- and post-exercise rapid-acting insulin reductions	25% pre-exercise and 50% post-exercise rapid-acting insulin dose preserved glycemia and protected patients against early-onset hypoglycemia (8 h)
Schiavon *et al*[233]	2013	In silico optimization of basal insulin infusion rate during exercise	A decrease in basal insulin by 50% starting 90 min before exercise and by 30% during exercise is safe and effective for glucose control
Danne *et al*[234]	2014	PLGM (suspension of insulin delivery based on predicted sensor glucose values)	PLGM may decrease the severity of hypoglycemia above that already established for algorithms that use a threshold-based suspension
Campbell *et al*[235]	2015	Combined basal-bolus insulin dose reduction and carbohydrate feeding strategy following exercise	Reducing basal-bolus insulin by 20% (80%) protected from nocturnal hypoglycemia for 24 h post-exercise
Cherubini *et al*[236]	2019	PLGM system during exercise	Effective for avoiding hypoglycemia during and after exercise, regardless of the thresholds of PLGM used
Moser *et al*[237]	2019	Oral administration of carbohydrates during moderate-intensity exercise	Pre-exercise BG levels determine the amount of orally administered carbohydrates during exercise to maintain euglycemia
Zaharieva *et al*[238]	2019	Basal rate reductions set 90 min pre-exercise *vs* pump suspension at exercise onset	50%-80% Basal rate reductions set 90 min pre-exercise improved BG control and reduced hypoglycemia risk during exercise better than pump suspension at exercise onset
Moser *et al*[239]	2019	Reduction in insulin degludec dose (75% IDeg dose *vs* 100% IDeg dose)	Reducing the usual IDeg dose by 25% led to more time spent in euglycemia with small effects on time spent in hypo- and hyperglycemia

Zaharieva *et al*[240]	2020	Insulin pump connected (pump on) *vs* pump disconnected (pump off) during high-intensity exercise	No significant differences in BG concentrations during 40 min of intermittent high-intensity exercise

CSII: Continuous subcutaneous insulin infusion; BG: Blood glucose; RT-CGM: Real-time continuous glucose monitoring; PLGM: Predictive low glucose management.

CONCLUSION

As T1DM is rising globally, PA is recommended as evidence shows that PA can control the burden of disease. The benefit of PA in T1DM is a significant component in its management. It is recognized as having beneficial effects and is key to a healthy lifestyle as well as the management of T1DM. PA can be considered an efficient and inexpensive non-pharmacologic tool for the management of T1DM in addition to insulin therapy. It has significant health benefits such as improves BG control, physical fitness, endothelial function, insulin sensitivity, well-being, and the body defense system. In addition, it reduces the blood lipid profile, insulin resistance, CVDs, insulin requirements, BP, and mortality associated with T1DM.

Overall, ideal glucose control is of principal significance in T1DM including during PA. Previously, it is challenging to prevent the hazards associated with PA in these individuals. Hypoglycemia and fear of hypoglycemia are the greatest challenges in T1DM individuals engaging in PA and can limit suitable glycemic control in these patients. However, a better understanding of energy metabolism and homeostasis has made it possible for individuals with diabetes to take part in exercise. In addition, several strategies have been identified to make PA more suitable for T1DM individuals. In particular, improvements in glucose monitoring technology and the availability of other interventional approaches during PA have further contributed to the feasibility of exercise programs for these subjects. There are also strategies for preventing exercise-induced hypoglycemia during and after exercise.

The present review may help health professionals to encourage PA as part of the management of individuals with T1DM. It is also recommended that PA can be performed carefully with reference to diabetes guidelines. Therefore, health professionals in clinical practice should inform and encourage patients with T1DM to manage exercise-induced hypoglycemia. Furthermore, in-depth knowledge of factors such as gender, therapy, glycemic control, training level and characteristics of the exercise performed will allow the development of individualized strategies to minimize the risk of hypoglycemia as the glycemic response to exercise depends upon these factors.

Moreover, health professionals should distinguish between hyperglycemia induced by HIIT and the concern of hypoglycemia-related to less intense forms of exercise during patient counseling for T1DM. Also, this should be clearly set out in practice guidelines. Patients and health professionals should be aware of the degree and duration of post-HIIT hyperglycemia and the potential benefit of an insulin correction bolus. Support for patients on how better to control their BG after exercise could encourage these patients to be less fearful of exercise-induced hypoglycemia and participate in regular PA. Clear-cut quantitative approaches to prevent exercise-induced hypoglycemia are desired to allow patients to engage in regular PA and enjoy its beneficial aspects. It is essential to consider the potential alterations in exercise responses that may occur in T1DM and not to judge this activity as harmful, but these alterations can reduce its full beneficial health effects. Lastly, the present review also provides suggestions for the future direction of research on the types of exercise, duration, and intensity recommended for T1DM patients, considering the individual's factors that could be detrimental to the response that occurs during and after exercise.

FOOTNOTES

Author contributions: The author made substantial contributions to conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and making critical revisions related to important intellectual content of the manuscript; and final approval of the version of the article to be published.

Conflict-of-interest statement: The author declare no conflicts of interest in this work.

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S-Editor: Zhang H
L-Editor: Webster JR
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 185-202

DOI: 10.4239/wjd.v13.i3.185

ISSN 1948-9358 (online)

REVIEW

Maternal low protein diet and fetal programming of lean type 2 diabetes

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Nie XQ, Saisho Y

Received: September 28, 2021
Peer-review started: September 28, 2021
First decision: December 4, 2021
Revised: December 30, 2021
Accepted: February 10, 2022
Article in press: February 10, 2022
Published online: March 15, 2022

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Abstract

Maternal nutrition is found to be the key factor that determines fetal health *in utero* and metabolic health during adulthood. Metabolic diseases have been primarily attributed to impaired maternal nutrition during pregnancy, and impaired nutrition has been an immense issue across the globe. In recent years, type 2 diabetes (T2D) has reached epidemic proportion and is a severe public health problem in many countries. Although plenty of research has already been conducted to tackle T2D which is associated with obesity, little is known regarding the etiology and pathophysiology of lean T2D, a variant of T2D. Recent studies have focused on the effects of epigenetic variation on the contribution of *in utero* origins of lean T2D, although other mechanisms might also contribute to the pathology. Observational studies in humans and experiments in animals strongly suggest an association between maternal low protein diet and lean T2D phenotype. In addition, clear sex-specific disease prevalence was observed in different studies. Consequently, more research is essential for the understanding of the etiology and pathophysiology of lean T2D, which might help to develop better disease prevention and treatment strategies. This review examines the role of protein insufficiency in the maternal diet as the central driver of the developmental programming of lean T2D.

Key Words: Type 2 diabetes; Maternal low protein diet; Fetal programming; Lean diabetes; Developmental origin of health and disease

Core Tip: This is to review the role of maternal low protein diet and its metabolic impact on the offspring leading to lean type 2 diabetes.

Citation: Vipin VA, Blesson CS, Yallampalli C. Maternal low protein diet and fetal programming of lean type 2 diabetes. *World J Diabetes* 2022; 13(3): 185-202
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/185.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.185

INTRODUCTION

Type 2 diabetes (T2D) is a metabolic disease, which is rapidly increasing among the human population both in developed as well as in developing countries. Diabetes is divided into four major categories: type1, type2, gestational, and other specific diabetes mellitus[1]. As per the national diabetes statistics reports in 2020, 10.5 % (34.2 million) of the United States population are diagnosed with some form of diabetes and 34.5% (88 million) of adults have pre-diabetes. T2D constitutes 90%-95% of all diabetes cases in the United States[2]. The hallmarks of T2D are insulin resistance and insulin deficiency. In most cases of T2D, the etiology of insulin resistance and insulin deficiency can be traced back to obesity and lifestyle aspects.

Scientists from all over the world have spent a great deal of time and effort to understand the causes and consequences of obesity-induced T2D. However, the number of non-obese/lean T2D cases has also dramatically increased globally and especially in Asia and other developing countries. Recent estimates show that 10%-20% of T2D patients are not obese[3]. Although the etiology is not clearly understood, lean T2D is clustered under the umbrella of T2D for patient care. Interestingly, clues from various studies indicate that lean T2D is often observed in the populations where the fetus is exposed to malnutrition during the intrauterine period or early childhood[3-7]. Adequate birth weight and size of the newborn are often considered as indicators of appropriate fetal growth rate and optimal *in utero* environment[8-10]. In contrast, deprivation of nutrition during fetal development is often marked by low birth weight and it is linked with adult-onset of metabolic diseases such as T2D[11].

Historically, observational studies from the different parts of the world firmly indicate that the individuals exposed to *in utero* malnutrition due to famine were more prone to the hyperglycemic condition in adult life compared to those who are not born during a famine[12]. For instance, the cohort exposed to Dutch famine (1944-45) *in utero* were more prone to glucose intolerance than the non-exposure cohort[13]. Similarly, data associated with the Ukrainian famine of 1932–33 have exhibited a higher incidence of T2D among the people born in the famine-affected region than in the regions where no famine was reported[14]. The link between Australian famines and T2D was studied and analysis showed a positive correlation with three years of famine and an increased number of T2D among those who were born during the famine years[12]. Further, Li *et al*[15] reported that people who were exposed to the Chinese famine in the fetal stage during 1959-1961 were more prone to hyperglycemia and T2D in their adult life, compared to those who were born after this period.

Recent studies indicate that adverse *in utero* nutrition could cause lean T2D later in life among certain ethnic and socio-economical groups, and people with certain lifestyles. Studies from India[16] (up to 26%) and Caribbean islands[17,18] (5%) report the predominance of lean T2D population. A study on American minorities showed that 13% of T2D patients are lean[4,16] with a fivefold higher incidence in Asians[4]. These observations clearly show that not all diabetics are obese and obesity does not necessarily cause T2D[3,16,19]. With > 42 million Americans experiencing food insecurity, it is a major problem even in the United States especially among the economically disadvantaged[20]. WHO estimates that 1.1 million children had ≤ 2 standard deviations for weight for height ratio (an index of protein-energy malnutrition) in the United States[21]. A recent German study shows that 38% of pregnant women did not consume enough protein[22]. With vegetarian and vegan diets gaining popularity worldwide, low protein intake is more prevalent as these diets are often low in protein[23,24]. Vegetarian mothers consume low protein diet[25,26] and give birth to children with lower birth weights, thus making them susceptible to T2D[26-28].

This atypical diabetic phenotype is known by various names such as Jamaica type diabetes, metabolically obese normal weight (MONW) diabetes, malnutrition-related diabetes mellitus, phasic insulin-dependent diabetes, tropical diabetes, mixed onset type diabetes, J, Z, M or type 3 diabetes, and ketosis resistant growth onset type diabetes[3,17,18,29-33]. This concept of MONW individuals was first proposed in 1981[29] with subsequent validations in animal and human studies[34-37] and the existence of lean T2D has been observed for decades but the etiology and pathophysiology of lean T2D are poorly understood.

The most accepted and validated hypothesis that explains the link between early nutrition and metabolic diseases in adulthood was proposed by David Barker is called, 'thrifty phenotype hypothesis'

[11,38]. This hypothesis explains how impaired *in utero* nutrition availability results in compromised fetal growth and programs subcellular and metabolic effects in the developing fetus. Further, the hypothesis suggests that the metabolic fate of an individual predisposed to T2D is decided at the early developmental stage and thus attempts to explain why a sub-population of individuals born with low birth weight are more prone to lean T2D compare to normal birth weight individuals[11]. Various subsequent studies have confirmed the reproducibility and epidemiological evidence for the 'thrifty phenotype hypothesis'[39].

In addition, studies based on this hypothesis showed the importance of a sufficient amount of protein in the maternal diet for the development of the fetus and the risk of diseases in adulthood[40]. Although overall well-balanced nutrition is essential for a developing fetus and a healthy offspring, the role of protein is vital in the developmental programming paradigm[41]. A low protein diet is well known to cause various programming effects leading to metabolic disorders in adulthood. Low protein or vegetarian diets are consumed due to various reasons such as poverty, famine, lack of availability, cultural, religious, or moral reasons, personal preference, *etc*. Although these are common in the developing world, the recent popularity of vegetarian and vegan diets in the developed world is also an important paradigm to be considered. The emerging popularity of vegetarian and vegan diets among the maternal population might compromise growing fetuses, as the amount and bio-availability of proteins are found to be inadequate from plant sources[23,42]. A low protein diet during gestation is often connected with compromised renal function and impaired glucose metabolism[7]. However, the mechanistic basis and the exact patient phenotype of the maternal low protein associated with lean T2D are not well understood. Therefore, a clear understanding of the epidemiological and clinical features of lean T2D is essential to the prevention or treatment strategies.

PATHOPHYSIOLOGY OF LEAN T2D

T2D is a complex metabolic disease with a spectrum of presentations. It is therefore essential to understand the pathophysiology of the disease to offer appropriate prevention and treatment strategies. The pathophysiology of lean T2D is not well defined, although we and others have considered them as a separate subset of T2D[3,43,44] body mass index (BMI) is widely used as a tool to classify T2D patients. Patients with a BMI greater than 25 are considered to have obese T2D[45]. In contrast, the majority of lean T2D patients have a BMI of less than 25 but they have several metabolic characteristics associated with obesity[3]. Observational studies in humans and experiments in rodents suggested that the various environmental and genetic factors could contribute towards the lean T2D phenotype[3,46]. Poor *in utero* nutrition during fetal development is considered to be the main driver of lean T2D onset[44,46]. We have shown using a novel rat model that the maternal low protein diet is one of the critical causes of the lean T2D phenotype[43].

Although the genetic factors may vary among the different populations, genetic predisposition to fragile beta cells was found to be common in lean T2D patients. The rapid beta-cell apoptosis is the major pathophysiological characteristic of lean T2D compared to elevated insulin resistance in obese T2D[47]. Another interesting aspect that is noticed in this population is the prevalence of truncal obesity. Insulin sensitivity and insulin response are varied among the different ethnic groups (African, Caucasian, and East Asian), and East Asians have more vulnerable beta cells which make them more prone to T2D[48]. Several studies on the South East Asian population have shown that lean T2D patients have central obesity or elevated visceral fat deposits[49]. Even though lean T2D patients have lesser hyperglycemic values, their hemoglobin A1c levels are significantly higher than their obese counterparts[5,44]. Further, the onset of lean T2D is reported at an early age than the obesity-associated T2D[3]. Lean T2D patients showed a significantly lower incidence of hypertension and cardiovascular diseases compare to obese T2D patients but are more susceptible to peripheral neuropathy[3,5]. Apart from environmental and hereditary factors, socioeconomic background is found to be an important aspect of lean T2D prevalence[10,50]. Several studies have reported an inverse relationship between T2D and socioeconomic status[51]. The National Health and Nutrition Examination Survey data indicated this relationship of poverty and higher incidence of T2D among African and Mexican origins in the United States[52]. The Chicago cohort study further showed the prevalence of lean T2D among this minority community[4].

The two crucial characteristic features of developing countries, which make them more vulnerable to lean T2D, are a rapid shift in lifestyle and impaired nutrition. Studies from India have reported the escalating number of lean T2D cases across the country, especially, the urban population of India[53-55]. Similarly, Alemu and the group reported the increased number of lean T2D like cases in the urban population of sub-Saharan Africa[56].

GESTATIONAL LOW PROTEIN PROGRAMMING AND SEX DIFFERENCES IN LEAN T2D

Sex differences in fetal development can be observed as early as the pre-implantation phase[57]. There are major *in utero* differences between the sexes in growth and metabolic parameters leading to a faster fetal growth in males when compared to females. These differences are attributed to the genes expressed by sex-chromosomes and the actions of sex hormones[57-59]. In addition, differences in the incidence of T2D can also be attributed to the differences in the leptin and insulin sensitivity between sexes[59,60]. These metabolic hormones are influenced by the *in utero* nutritional environment[61]. Many studies have found a link between T2D and maternal low protein diet[43,59,62,63]. Further, as the nutritional environment often regulates the epigenetic machinery, any change *in utero* nutritional status may cause permanent alterations in the fetal gene expressions[64].

Our research using a lean T2D rat model indicates a clear sex difference in glucose homeostasis with females developing glucose intolerance earlier in life with faster disease progression than males[43,65]. These animals also showed differential regulation of gluconeogenesis and glycogenolysis as a result of gene expression changes in key genes involved in glucose metabolism[65-68]. Sex difference in hepatic genes associated with glucose homeostasis such as phosphoenolpyruvate carboxykinase (PEPCK) and 11β-hydroxysteroid dehydrogenase type 1 were observed even in low protein programmed fetuses[69]. Similarly, low protein programmed mice offspring were found to have lower birth weight with more glucose intolerant than the controls[70]. In this study, maternal low protein diet activated the visceral adipose tissue neuropeptide Y-Y2 receptor system in female offspring but not in male offspring, which increased abdominal adiposity and insulin resistance in female offspring[70]. This study indicates the importance of neuropeptide Y-Y2 receptor as a potential sex-specific marker and mediator of metabolic programming[70].

In the last decade, various studies have shown the importance of mitochondrial health and its relationship with glucose homeostasis in low protein programming. Zambrano and group have found maternal low protein diet-induced insulin resistance in male Wistar rats; however, females were responsive to glucose[71]. This study, further, suggested that elevated mitochondrial dysfunction in the pancreatic islets of adult male rats might be the mechanism that leads to insulin resistance[71]. Likewise, male offspring of low protein diet-fed mothers showed higher ROS production and impaired electron transport chain function in the mitochondria of the pancreatic islets when compared to female offspring indicating mitochondrial incompetence in males could predispose them to T2D[72]. Similarly, the sex dependent fetal programming in glucose metabolism was also reported *in utero* low protein programmed piglets. In this study, hepatic gluconeogenesis in newborn male piglets was negatively affected by the maternal low protein diet during pregnancy[73]. Epigenetic changes in the promoter region of the glucose-6-phosphatase gene were sex-specific and resulted in T2D in adult male pigs[73]. In addition, maternal low protein diet diminished liver mtDNA copy number in males and altered the OXPHOS protein expression by the combined binding action of glucocorticoid receptor and methylation of on the hepatic mtDNA promoter, which effect the mtDNA replication and gene expression levels[73,74].

Studies in humans suggest greater prevalence and impact of lean T2D in males than females. Many studies have indicated that women are physiologically inclined to have better insulin sensitivity than men[75-77]. Estrogen has a protective role in insulin sensitivity and glucose homeostasis by the inhibition of Foxo1 though activation of ERα-PI3K-Akt signaling[78]. Another crucial way estrogen protects women from insulin resistance is through mitochondrial biogenesis, as testosterone reduce mitochondrial proliferation[79]. The male preponderance of lean T2D was evident from the studies conducted in India, where more than 60% of lean T2D patients were men. Although the exact causes of sex differences are not clearly understood, it is suggested that the differences observed in this study may be due to predominant male exposure to oxidative stressors such as smoking and alcoholism[3,80].

Another interesting aspect to consider is the role of folate. Folate is routinely given to pregnant women throughout the world to prevent neural tube defect. Recent studies show that excessive folate can also have negative consequences at least in certain populations, ages and ethnicity/genetic background[81-83]. One study in Indian population shows that it can lead to insulin resistance[7]. The authors primarily attribute this to the deficiency of vitamin B12 which is primarily present in animal protein. Rat studies from our group showed that folate offered some protection in low protein programmed offspring by compensatory hyperinsulinemia but make insulin resistance worse in males [84]. Although the mechanism of this sex dependent folate action in insulin resistance is not known and warrant further research, it is important to note how folate may have sex dependent effects and this may also hold a clue in the sex differences that are observed in the human population.

ANIMAL MODELS

Considering the ethical and technical limitations in conducting impaired maternal nutrition and developmental programming studies in humans, various animal models that mimics several aspects of developmental programming have been developed. Due to the shorter lifetime and availability of

genetic tools, a substantial amount of research is presently focused on developing clinically reliable rodent models of developmental programming.

To achieve a low protein diet model, the majority of studies followed a diet that has around a 50% reduction of total protein in the diet formulation[43,63,85-87]. However, most of the investigations conserved the isocaloric nature of the diet by manipulating macronutrient proportions by various lipid and carbohydrate ratios[88-93]. Although preferred protein, carbohydrate, and lipid ratio are varied among different research groups; a single research group often stick to one specific diet regimen[67,91,94-97]. The other central deviation apparent among the different low protein models is the timing and duration of the maternal diet management. Majority of the studies have started giving low protein diet from the first day of the pregnancy and continued throughout pregnancy or lactation, although some studies initiated the low protein diet before pregnancy or in some cases in a specific period of *in utero* growth[65,86,98-102]. The main aim of these refined diet manipulations is to develop a metabolically compromised adult offspring[103]. Moreover, many studies have succeeded in mirroring low birthweight and catch-up growth pattern, which is considered by many as a hallmark of the developmental origin of metabolic disease[104-108]. Pups from maternal low protein mothers weigh less compared to those from control diet-fed mothers. The differences in birth weight disappeared once the mothers were fed with a normal diet or pups were cross-fostered with control mothers. However, the weight differences were permanent, when the maternal low protein diet was continued throughout the weaning[43,100,109]. In addition, due to the variation in macronutrients ratio and the time regime of the diet, the adult metabolic phenotypes reported by various groups are also varied. Insulin resistance, obesity, cardiovascular diseases, and dyslipidemia are the major clinical disorders observed in these models[104,110-113]. A comprehensive list of different low protein programming animal models used are summarized in Table 1.

PHYSIOLOGICAL EFFECTS AND MECHANISMS

Many animal models based on a low protein diet have been successful in capturing the phenotypic characteristics of fetal programming of adult metabolic diseases. However, the exact mechanism that leads to these metabolic diseases is not well studied. The dominant hypothesis in the field of developmental programming of adult diseases attribute that the fetal epigenome play a central role. This hypothesis postulates that epigenome is reprogrammed as an adaptation in response to a low protein diet, the associated low birth weight, and the catch-up growth. A recent study in Japanese adults indicates that the reduced beta cell mass in low-birth-weight individuals is directly associated with the future development of T2D[114]. Although the epigenome is prone to modification throughout the lifetime, *in utero* developmental period was found to be the most vulnerable time to be dysregulated by stressors[115].

Several studies have reported various key genes that are epigenetically modified as a result of developmental programming. For instance, the transcription factor Hnf4a was found to be epigenetically regulated during gestation, and the maternal diet-induced changes in the expression of this gene can cause T2D in adulthood[116]. Similarly, glucose transporter 4 (GLUT4) expression in skeletal is epigenetically controlled by maternal diet during early development and the impaired gene expression often resulted in peripheral insulin resistance[117]. Even though different biological mechanisms might contribute to fetal programming of lean T2D, many recent studies are indicating epigenetic changes as a potential single important driver of the fetal programming effects[118]. Low protein diet exposure during pregnancy in animals exhibited changes in methylation in promoter regions of genes involved in the glucose homeostasis pathway thereby, affecting the gene expression either directly or indirectly [119]. In recent years, many experimental studies in animals and observational studies in humans show that the epigenetic changes associated with gestational low protein are the main regulatory forces mediating the T2D phenotype[118,120]. Changes in the fetal epigenome often mirror the unique *in utero* environment of the fetus. Epigenetic changes due to gestational low protein arise through the methylation of cytosine in CpG Island present in the promoter region of particular genes, histone protein modification by acetylation, and regulation microRNAs by post-transcriptional modification. The chromatin structure and expression of a specific gene are regulated through DNA methylation in association with histone modifications[121].

A study in pigs found a significant decrease in glucocorticoid receptor binding to the glucose-6-phosphatase (G6PC) promoter which was accompanied by hypomethylation of the G6PC promoter in association with gestational low protein diet[74]. As G6PC is one of the crucial enzymes in glucose homeostasis that catalyzes gluconeogenesis and glycogenolysis, epigenetic changes in the promoter region might contribute to the onset of hyperglycemia[74]. Further, this impaired maternal diet-induced reduction of mtDNA copy number and methylation of mtDNA promoter often leads to changes in OXPHOS gene expression. This may predispose to insulin resistance in adult offspring considering the importance of hepatic mitochondrial OXPHOS activity in glucose homeostasis[73].

Similarly, using maternal low protein programmed rats, Lillycrop *et al*[8] established that the hepatic PPARα promoter and glucocorticoid receptors were hypomethylated *in utero* and these epigenetic

Table 1 Summary of key animal models used to investigate the maternal low protein associated insulin resistance and glucose intolerance

Animals	Diet regimen	Age of pups	Sex	Observations	Ref.
Sprague-Dawley rats	6% protein, -12 to 43 d	12 wk	Females	Sirt3 dysfunction in skeletal muscle	[138]
Sprague-Dawley rats	10% protein, 2 to 21 d	Newborn	Males	Increased *Igf* gene expression	[148]
Sprague-Dawley rats	8% protein, 1 to 43 d	17 wk	Males	Lower fasting insulin and HOMA	[85]
Wistar rats	6% protein, 1 to 21 d	11 wk	Females	Insulin resistance and glucose Intolerance	[103]
Wistar rats	6% protein, 1 to 43 d	3 wk	Both	Compromised β-cell structure and function	[163]
Wistar rats	7% protein, 1 to 120 d	16 wk	Females	Higher glucose tolerance and insulin responsiveness	[98]
Wistar rats	8% protein, 1 to 43 d	12 wk	Both	Impaired gluconeogenesis, glucose handling and liver structure	[141]
Wistar rats	8% protein, 1 to 43 d	11 wk	Females	Insulin resistance and glucose Intolerance	[190]
Wistar rats	8% protein, 1 to 21 d	12 wk	Males	Epigenetic regulation of Hnf4a in islets	[116]
Wistar rats	8% protein, 1 to 21 d	12 wk	Both	Altered mitochondrial function in islets	[72]
Wistar rats	8% protein, 1 to 21 d	12 wk	Both	Structural alterations and changes in glucokinase expression in liver	[141]
Wistar rats	8% protein, 1 to 21 d	Fetal Day 21.5	Both	Altered IGF axis and proliferative capacity of liver	[140]
Wistar rats	9% protein, 1 to 20 d	Fetal Day 20	Both	Defective hepatic glucose homeostasis	[69]
Wistar rats	10% protein, 1 to 21 d	4 wk	Both	Impaired hepatic gene expression	[8,122]
Wistar rats	10% protein, 1 to 43 d	15 wk	Both	Modified glucose metabolism and insulin resistance	[71]
C57BL/6J mice	9% protein, 1 to 39 d	8 wk	Both	Impaired glucose metabolism, miR-15b up-regulation	[63]
C57BL/6J mice	8% protein, 1 to 21 d	3 wk	Both	Altered PPAR signaling, insulin resistance and glucose Intolerance	[87]
C57BL/6J mice	8% protein, 1 to 19 d	Newborn	Both	Altered mitochondrial genes expression in liver and skeletal muscle	[89]
Mice	8% protein, 1 to 40 d	21 wk	Both	Increases abdominal adiposity and glucose intolerance	[70]
Pig	6% protein, -18 to 113 d	Newborn	Both	Affected mitochondrial OXPHOS and glucose-6-phosphatase in liver	[73,74]

changes were persistent in adulthood. Further studies demonstrated reduced Dnmt-1 expression and its role in epigenetic changes of glucocorticoid receptors[73,95,96,122]. Moreover, epigenetic changes in the promoter region of PEPCK were found to be the drawing force for impaired glucose homeostasis in animals[123,124]. Anandwardhan and colleagues reported a decreased number of (pro) insulin 2 gene transcripts in the pancreas of low protein *in utero* programmed rats, due to the histone modification in the promoter region of the insulin 2 gene[125]. Moreover, these epigenetic changes are potentially engaged in the trans-generational transmission of the induced phenotype[122,124,125].

Recently, Goyal and group demonstrated that the epigenetic modifications by miRNA, small non-coding RNAs consists of 20–22 nucleotides, is one of the molecular mechanisms of maternal low protein-induced T2D[119]. Results from maternal low protein programmed mice found reduced beta-cell mass and insulin levels in the pancreatic islets of the programmed offspring due to the increased expression of miR-15b. As the activities of cyclins are negatively regulated by the presence of miR-15b, the up-regulation of this miRNA may inhibit pancreatic beta-cell proliferation, consequently, stem to T2D phenotype[63]. A microarray study also demonstrated elevated expression of miR-615, miR-124, miR-376b, and decreased expression of miR-708 and miR-879 in maternal low protein programmed mice, which were associated with degenerated metabolic health of the offspring from the weaning age [126].

Apart from the epigenetic changes, maternal malnutrition is the major reason for low birth weight in newborns. Children who are small for gestation age and showed catch-up growth during the early age of development appeared to be more insulin resistant compared with normal-weight children[127]. Moreover, several studies have shown epigenetic changes due to gestational diet-induced fetal programming adult diseases in these offspring[108,128,129].

Even though little is known about the mechanism of programming, the secondary effects of fetal programming and their mechanisms are well studied. For example, various organ systems that play vital roles in the metabolism, and how they are affected by the developmental programming of T2D are well characterized. *In utero* low protein exposure causes long-lasting structural and functional changes in metabolically active organs includes skeletal muscle, liver, pancreas, gonads, and brain.

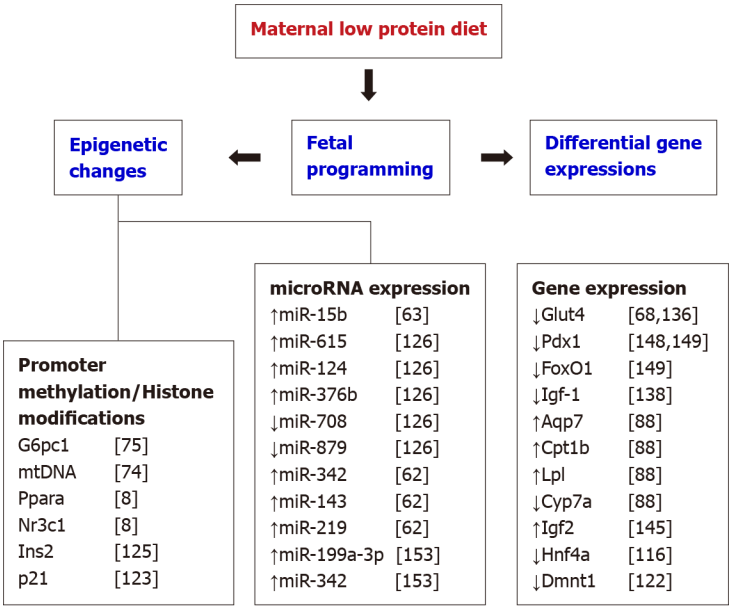
The *in utero* environment is crucial in the development of skeletal muscles, and the muscles growth is determined by the number, size, and type of muscle fibers formed during fetal development[130,131]. Maternal undernutrition affects the quality and quantity of skeletal muscles and stem cell activity[132-134]. A maternal low protein diet during gestation affects the normal proliferation and differentiation of bone marrow stem cells and satellite cell function[134,135]. Therefore, imperfections of skeletal muscles development during fetal development are often deleterious to normal muscle functions in adulthood [133]. Studies using maternal low protein diet-based animal models have reported lower expression of GLUT4 and mitochondrial dysfunction in skeletal muscles of low protein offspring[66,67,136-138]. As skeletal muscle functions as one of the main sites for peripheral glucose disposal, functional or structural changes of the myofibrils leads to insulin resistance and glucose intolerance[139].

Similarly, low protein-induced developmental programming caused functional and structural changes in the liver[140,141]. The expression of genes associated with oxidative phosphorylation and glucose metabolism were altered in the liver. Further, *in utero* low protein exposed rat fetuses showed the altered structure of the liver with decreased proliferation of hepatocytes[142-146]. These animals also had altered hepatic lipid metabolism and hepatic desaturase activities, which may account for fetal growth retardation and insulin resistance[94,147]. In addition, a maternal low protein diet also induces epigenetic changes in methyltransferase machinery resulting in altered epigenetic regulation in the liver [148]. Although further studies are warranted, it is clear from the existing studies that developmental programming induced by a low protein diet affects hepatic structure and function and this may, in turn, make them susceptible to impaired glucose metabolism[141,145,149].

The ability of the pancreatic β-cell to secret insulin is dependent on its structural and functional integrity along with the nutritional availability[150]. Consequently, protein deficiency in the maternal diet is a definite contributor to reduced insulin secretion and decreased β-cell proliferation in low protein programmed animals[151]. The reduced islets area and β cell number are mainly due to the downregulation of genes *FoxO1* and *Pdx1* genes, or altered expression of Reg1 pathway genes[151-155]. Epigenetic regulation of Hnf4a expression and expression of microRNAs such as miR-15b, miR-199a-3p, and miR 342, and signaling of mTOR in islets of the progeny also found to be associated with low protein-induced beta-cell dysfunction[62,63,156]. Further, a maternal low protein diet demonstrated greater β-cell apoptosis rates and deviates the equilibrium of islet's apoptosis and replication in the offspring[157-159]. The pancreatic islet cells of these offspring exhibited greater oxidative stress and mitochondrial dysfunction[72,160]. Consequently, lower β-cell reserve, β-cell dysfunction and impaired mitochondrial function in islets may drive towards T2D later in life[62,72,86,155]. With the multiple pathways controlling β-cell functions are modulated by maternal low protein, it is reasonable to hypothesize that the low protein exposure predisposed the offspring to lean T2D[127]. A list of key genes involved in low protein programming is compiled in Figure 1.

A balanced *in utero* nutrition is essential for the normal development of the reproductive system. Epidemiological studies in humans and experimental in studies animals show the low protein/unbalanced diet *in utero* severely impacts the development of reproductive organs, sexual maturation, and reproductive function in the offspring[161-164], resulting in decreased testis weight, reduced Sertoli cell numbers, and late-onset of spermatogenesis in males[164-166]. Moreover, the classical male fertility markers, sperm count, and serum testosterone were also diminished in the offspring of low protein exposed mothers[163,164,167]. Similarly, the low protein programmed female offspring were found to be with compromised follicle development and follicle health[168,169]. The numbers of primordial, primary, and secondary follicles were significantly reduced along with abnormal estrous cycle and redox homeostasis[170-172]. The thyroid hormone production and hypothalamic-pituitary-gonadal axis are also found to be affected by maternal low protein diet[173,174]. The impaired reproductive function of offspring may be due to the altered expression of genes associated with steroidogenesis, folliculogenesis, and steroid hormone receptors in gonads[175-178]. In addition, changes in the hypothalamic-pituitary-gonadal axis to low protein may have adverse effects on the normal development and function of gonads[168,179].

The hypothalamus of the brain plays a critical role in glucose homeostasis by controlling hepatic glucose production and peripheral glucose utilization. Therefore, functional, or structural alteration of hypothalamic neurons may often lead to the onset of T2D[180,181]. The low protein programmed progenies have exhibited structural and functional changes in the neuronal centers, hypothalamic nuclei which regulate metabolism and body weight[102,181]. In addition, maternal low protein can also differentially affect the hypothalamic-pituitary-gonadal axis depending on the timing of the impaired nutrition. Early gestational nutrition impairment has been shown to make the pituitary more sensitive to GnRH, resulting in reduced reproductive function[182]. Further, it also alters hypothalamic-pituitary-adrenal axis function by deregulating corticosterone-inducible enzymes and associated enzyme receptors[183]. Other reports also show that brain sparing may not be as effective during *in utero* low protein exposure leading to compromised brain development in the offspring along with long-lasting deterioration in cognitive and motor functions[184,185].



DOI: 10.4239/wjd.v13.i3.185

Figure 1 Key gene expression and epigenetic changes observed in different maternal low protein studies.

PREVENTION AND TREATMENT

Insulin resistance and glucose intolerance are the cardinal signs of T2D, and if not prevented, they may lead to severe diabetes complications later in life. Hepatocytes, skeletal muscles, and adipocytes are the major insulin-dependent tissues that participate in the disposal of peripheral glucose. Thus, improving the muscle sensitivity towards insulin and enhancing hepatic glucose homeostasis, along with managing body weight are the central focus of T2D treatment strategies. Among the different drugs that have been prescribed for leanT2D management, metformin is a widely used drug for treating lean T2D along with nutritional and lifestyle modification[186]. Over the past two decades, various randomized control trials conducted in many ethnic groups showed unambiguously that the prevention is feasible by drugs or lifestyle modification[187-190].

Most of the research on treatment or prevention of T2D has been done with obese individuals or animal models, even though 10%-16% of all T2D people have normal BMI. In addition, majority of the studies on the molecular mechanisms of prevention and reversal of T2D were performed in Caucasians. Consequently, it is essential to include other ethnic groups such as Southeast Asian and Chinese populations, which are more prone to diabetes at lower average BMIs or lean T2D compared with white Europeans[191]. Regulating body weight is critical in the management of T2D associated with overweight or obese patients. However, in the case of leanT2D, it seems that leaner patients have severe beta-cell failure than normal-weight patients[4]. Presently, it is not clear that the achievement of lower body weight will help to prevent or reverse the special variants of T2D such as lean T2D[3].

The first trial associated with lifestyle modification and/drug therapy was started in China with a follow-up period of 23 years[192] and many other studies have followed since. Other studies include: the American diabetes prevention program outcome study[193]; the Finnish diabetes prevention study [194]; and the Indian short message service study[195] revealed the influence of lifestyle modification can persist long after the termination of the active phase of the trial. Although lifestyle modifications have been recognized to be very effective, safe, and ideal strategy for prevention, the effectiveness of relative risk reduction through these strategies exhibited some variations among different ethnic populations[192-196]. A study conducted among the impaired insulin tolerant lean Indian population found that lifestyle modification alone prevented the diabetes onset, regardless of relatively low BMI and highly insulin-resistant characteristics of the population[197].

Although the underlying pathophysiology of lean T2D is not completely understood, many studies using the maternal low protein model have shown potential prevention approaches[157]. The most promising approach among them is associated with one-carbon metabolism and the molecules involved in it. Some studies have reported the effectiveness of folic acid supplementation as a preventive treatment against the adverse effects of fetal programming[198-200]. Similarly, Burdge and team reported that the folic acid supplementation reversed the maternal low protein-induced phenotype epigenetically in the offspring when treated during the juvenile-pubertal period[201]. In contrast, our study reported a partial inhibition of gestational low protein-induced glucose intolerance only in female rats, when the maternal low protein diet was supplemented with folic acid from day 4 of the pregnancy until delivery[65]. Similar to our data, Lillycrop and colleagues also reported the inefficiency of folic

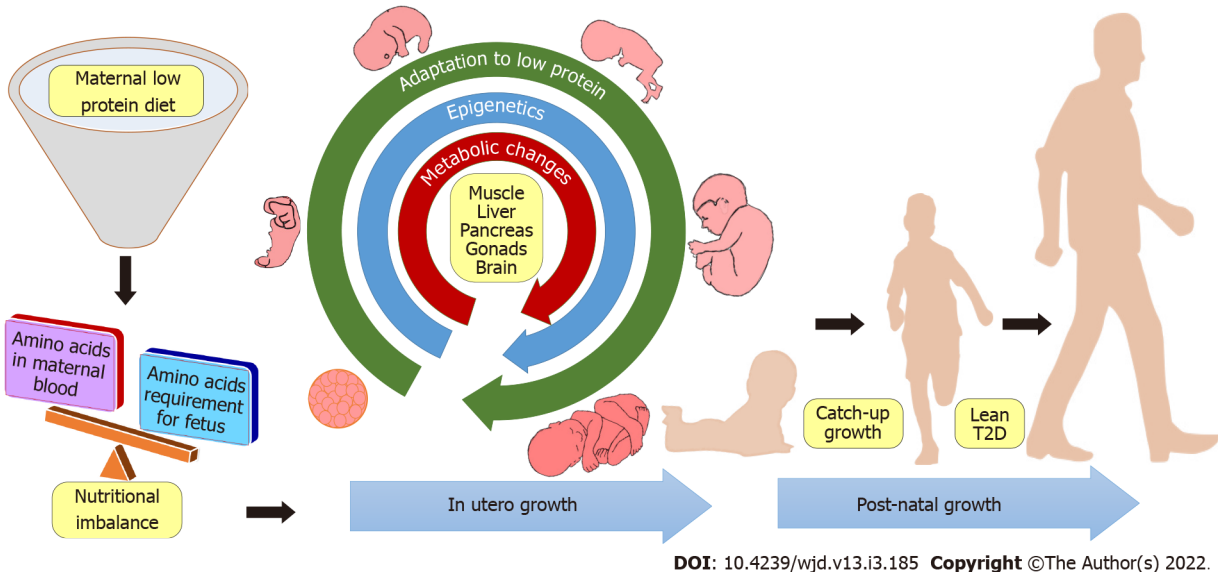


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Figure 2 Proposed mechanism of maternal low protein associated lean type 2 diabetes.

acid supplementation for the inhibition of gestational low protein-induced change in gene profile, although they found changes in the expression of genes associated with redox homeostasis[8]. An observational study from Pune, India (Pune Maternal Nutrition Study), noticed that when the mother was vitamin B12 deficient, high amount of folic acid intake was not enough to prevent the insulin resistance in the offspring[7]. However, the high protein to carbohydrate ratio in maternal diet was found to be effective in maintaining glucose homeostasis in the offspring[137]. Thus ensuring sufficient protein in the maternal diet is essential to prevent lean T2D.

CONCLUSION

In summary, lean T2D is a discrete subgroup of T2D with a set of specific clinical profiles. Atypical characteristics of leanness associated with insulin resistance needed to be dissected further for a better understanding of the etiology of the disease. As the progression of T2D may take many years in humans, the assessment and prevention studies with human subjects may also warrant many years. Therefore, the development of a well-defined animal model, which mirrors not only the pathophysiology of lean T2D but also the etiology of the disease, might be the most important step in this area of research. Nevertheless, there is a lack of a single animal model that can constitute all pathophysiological and etiological changes similar to humans. In addition, the severity of lean T2D is different between sexes, due to sex hormones and sex dependent expression of genes. Among different molecular mechanisms involved in the onset of lean T2D, the epigenetic underpinning of metabolism appears to be the most promising lead. Although the mechanism of developmental programming is currently not well characterized. With the current literature, it may be summarized that maternal low protein diet leads to diminished essential amino acids levels in the maternal circulation and consequently to the fetus. In such low protein environment, fetus is acclimatized and revises its growth and metabolic set points. This adaptation is thought to be due to the overall alteration of epigenetic and metabolic attributes of fetal energy homeostasis. Although these adaptations may be beneficial for the fetus, a nutritional mismatch with protein abundance in the adulthood often leads to metabolic derangements leading to diseases such as lean T2D. This concept is summarized in Figure 2. A better understanding of the molecular mechanisms of the disease may pave the way for more effective preventive and treatment strategies.

Obesity associated T2D is a serious public health problem throughout the developing and developed countries whereas nutritional deficiency especially protein deficiency is a major concern in under developed and developing countries. With studies showing a link between maternal protein consumption and T2D in offspring, it is essential to probe further and take action to avert a global crisis. Public health measures to alleviate poverty and access to nutritious and protein rich diet during pregnancy is essential to prevent lean T2D. Scientific understanding of the disease to prevent and treat T2D, along with effective health education and public policies can mitigate this growing global epidemic.

FOOTNOTES

Author contributions: Author Contribution: Vipin VA, Yallampalli C and Blesson CS had substantial contributions to conception, synthesis and survey of literature, and contributed to draft the manuscript.

Supported by the National Institutes of Health Grants, No. HL102866, HL58144 and DK114689.

Conflict-of-interest statement: Authors have no conflict of interest.

Country/Territory of origin: United States

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 203-212

DOI: 10.4239/wjd.v13.i3.203

ISSN 1948-9358 (online)

MINIREVIEWS

Can the management of depression in type 2 diabetes be democratized?

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Xiong YP, Zeng Y

Received: October 29, 2021
Peer-review started: October 29, 2021
First decision: December 27, 2021
Revised: January 7, 2022
Accepted: February 22, 2022
Article in press: February 22, 2022
Published online: March 15, 2022

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Abstract

Both type 2 diabetes and depression are common and are projected to increase. There is increasing evidence for a bidirectional relationship between the two. Diabetes is a risk factor for depression; contrariwise, individuals with depression are at greater risk of developing diabetes. They are a burden for both the individual and the society. Co-existent depression worsens diabetic control because of obesity, insulin resistance and the adverse metabolic effects of anti-diabetes medicines. In addition, compliance to lifestyle measures required for diabetes is also compromised such as following a specific diet, taking proper medications on time, getting metabolic parameters assessed and maintaining a sleep cycle. Depression occurs in many grades; mild depression is more common in diabetes than frank or full-blown depression leading to suicide. Unfortunately, there are not enough trained and accessible mental health professionals such as psychologists or psychiatrists to deal with the increasing burden of depression in diabetes. Therefore, alternate models for management of mild to moderate depression are required. There is evidence that a team-approach by employing health care assistants can lower the risk of cardiac risk factors. INtegrating DEPrEssioN and Diabetes treatmENT study was carried out to determine whether the team-approach using non-health care professionals could be effective in managing mild to moderate depression and to study its effects on metabolic parameters among subjects with type 2 diabetes mellitus. The international study, carried out in four independent centers in India assessed the impact of a trained but not qualified non-psychiatrist in coordinating and forming a fulcrum between the patient, the family and the consultant endocrinologist/diabetologist. The interventions were fine-tuned to be culturally appropriate by qualitative interviews before they began. It was shown that the outcomes of both depression and diabetes could be improved by the employment of a clinical care coordinator. It is possible to scale up the studies to wider geographical areas and health-care organizations.

Key Words: Insulin resistance; Bidirectional; Patient health questionnaire-9; Care-coordinator; Antidepressants; Integrating DEPrEssioN and Diabetes treatmENT study; Non-professional

Core Tip: Type 2 diabetes and depression frequently co-exist. The presence of one worsens the outcome of the other. There are insufficient qualified professionals to treat depression. The INtegrating DEPrEssioN and Diabetes treatmENT study has shown that care-coordinators, who are trained but not professionals in mental health care can integrate and liaison among the patient, the family and specialists in treating mild to moderate depression associated with diabetes. Deployment of care-coordinators improved the outcome of depression and diabetes. This proof-of-concept study can be expanded and if found useful, help in democratizing the management of depression in diabetes.

Citation: Sridhar GR. Can the management of depression in type 2 diabetes be democratized? *World J Diabetes* 2022; 13(3): 203-212
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/203.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.203

INTRODUCTION

The prevalence of type 2 diabetes is growing worldwide with depression rapidly following. Though it is possible that two common conditions can co-exist independent of one another, there is increasing evidence that diabetes and depression are related pathophysiologically, sharing a bi-directional relationship. When depression and diabetes coexist, the quality of life, compliance to treatment and outcomes are poor. Qualified specialists to manage diabetes and depression are in short supply. Therefore, innovative approaches are necessary to improve the outcomes of both. Based on published data between 1990 to 2016, it was estimated that among those aged 18-99 years, there were 451 million people with diabetes. By 2045, these were projected to increase to 693 million[1]. Of those with diagnosed diabetes, there is a greater prevalence in urban rather than rural (10.8% *vs* 7.2%) areas, and in high-income than in low-income countries (10.4% *vs* 4.0%)[2]. Mental disorders accounted for 13% of the global disease burden; major depression is projected to be the chief contributor to mental disorders by the year 2030[3]. Depression is commonly seen in other chronic illnesses also[4]. Multifactorial etiology of diabetes[5] and depression[6] requires multi-pronged management strategies.

COEXISTENCE OF DIABETES AND DEPRESSION

Diabetes mellitus is not a homogenous condition but results from a variety of pathogenic factors which are not always exclusive[7]. However, for clinical purposes, diabetes is classified into (1) Type 1 diabetes due to autoimmune destruction of the pancreatic β-cell leading to absolute insulin deficiency; (2) Type 2 diabetes mellitus having insulin resistance and a progressive loss of β-cell insulin secretion; (3) Gestational diabetes; and (4) Other specific causes leading to diabetes[8]. It is evident that psychological reactions differ in each of the different varieties of diabetes. In this presentation, management of depression is focused on type 2 diabetes, which is more common.

Twice as many people with diabetes are likely to have depression compared to the general population[9,10]. Hypertension, which is common in diabetes is associated with risk of depression and anxiety[11]. Resultantly the association of depression and diabetes has been the most commonly studied for the longest time[12]. A meta-analysis showed that compared to those with normal glucose tolerance, depression was more common in people diagnosed with diabetes; it was not high in those with pre-diabetes or those with normal glucose tolerance[13].

The number of prospective studies on the course of depression among people with diabetes is small; a meta-analysis of 11 follow up studies showed that type 2 diabetes subjects have a 24% increased risk of developing depression compared to controls[14]. Similarly, people with depression have a 32% increased risk for developing type 2 diabetes mellitus[15].

The grades of anxiety and depression associated with diabetes vary from subclinical depression to diabetes distress, which refers to emotional distress resulting from living with diabetes, a chronic non-remitting disease[16]. There are serious clinical implications when depression coexists with diabetes: The quality of life is impaired; the risk of morbidity and death is also increased. Operating factors include poor health care behavior which affects dietary habits, treatment, compliance to treatment,

motivation and productivity[16]. Long term diabetic complications are more common with comorbid depression[17]. Finally, the impact of combined diabetes and depression on quality of life is significant. Healthcare costs of managing type 2 diabetes associated with depression is higher than that of diabetes without depression[18]. Depression in type 2 diabetes can be treated[19], which improves the quality of life[17]. One must distinguish depression from diabetes distress. Diabetes distress is an emotional response to having diabetes, specifically the restricted lifestyle with having to follow self-management and the potential of complications in the long term[20]. Diabetes distress is associated with lessened self-care, and poorer emotional well-being, which, if left untreated may progress to severe depression[21]. Diabetes distress is far more common than clinical depression and is associated with poorer glycemic control[22]. The poor outcome is mediated in part by perceived control over diabetes such as one's innate ability to influence the course of diabetes[23].

Unlike the diagnosis of diabetes mellitus, depression is identified by clinical features such as episodes of lowered mood, reduced energy and decreased activity[8].

At the other end of the diabetes and depression spectrum is suicidality. Depression in persons with diabetes increases the risk of suicidality[24]. One must be aware of the risk factors for suicidal ideation and suicidal behavior, such as insulin administration, long duration of diabetes and poor glycemic control[25]. Identification and preventive measures are therefore essential in subjects with diabetes having depressive symptoms. Interventions must not only consider medical treatment, but also social factors associated with them[11], pointing to the need for integrated management processes.

COMMON PATHOGENESIS OF THE TWO CONDITIONS

Epidemiological studies have shown a bi-directional association between diabetes and depression[26]. Mendelian randomization studies have provided evidence that type 2 diabetes mellitus can cause depression: Single-nucleotide polymorphisms that predispose to diabetes predicted symptoms associated with depression[27]. Xuan *et al*[27] used 34 T2D risk genetic variants validated in East Asians as the instrumental variable (IV). An analysis using Mendelian randomization was carried out on 11506 participants from a prospective study. The diabetes genetic risk score (GRS) was built employing the 34 T2D common variants. The GRS was associated with depression even after adjusting for variables including age, sex, body mass index, current smoking and drinking, physical activity, education and marital status. A causal relationship was also found between genetically determined T2D and depression[27]. In addition, the stress associated with a new diagnosis of diabetes can precipitate depression[28].

The *common soil hypothesis* posits that factors common to both conditions could be the link for their association such as chronic inflammation, sedentary habits leading to obesity as well as vascular dysfunction[10]. Conceptually, the factors relating to both can be considered at two levels: *Behavioral* and *biological*[12]. Behavioral components include the burden of dealing with a chronic non-remitting disease and resultant poor lifestyle behavior. Sedentary lifestyle is a risk factor for depression[29], just as it is for obesity and diabetes mellitus. Biologically, hyperglycemia, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, chronic low grade inflammatory response and vascular dysfunction could all contribute. These are common to both diabetes and depression and may contribute to their co-existence[12]. There is evidence that behavioral and environmental factors are more responsible than genetic factors[12]. Vascular changes in small vessels supplying blood to the cerebral cortex are found in depression[30], although confirmatory studies are required. Brain-body dysfunction may contribute by impaired HPA regulation and by brain-gut microbiome axis[12]. Similarly, social stress can operate through epigenetic factors that activate the inflammatory response which is common to both diabetes and depression[15]. Use of some antidepressant drugs is also implicated in the risk of obesity, insulin resistance and diabetes mellitus[24,31].

Inflammatory changes, which occur in obesity, insulin resistance and diabetes mellitus occur in depression as *neuroinflammation*, involving activated microglia, astrocytes and oligodendroglia. These release mediators such as cytokines and chemokines, which when persistent, cause neurotoxicity[32]. Chronic inflammation in turn leads to insulin resistance and endothelial dysfunction, which has also been described in depression[32]. Hormonal components in women may contribute to gender differences in pathophysiological changes involving dysregulation of HPA and AN systems acting *via* immune and hemostatic pathways[33]. There is a flattening of the diurnal curve of the stress hormone cortisol which is associated with insulin resistance and could thereby play a role in the coexistence of diabetes and depression[34]. Along with abdominal obesity and insulin resistance, hypercortisolemia induces changes in glucocorticoid receptor-rich brain areas such as the hippocampus, amygdala and prefrontal cortex, where emotions and cognition are mediated[34].

Conceptually the relation between diabetes and depression can be considered in terms of (1) Psychological burden of a chronic disease such as diabetes predisposing the patients to depression and poor self-care behavior; (2) Diabetes and depression are coincidental, sharing common environmental and lifestyle factors; and (3) The cognitive behavioral construct attributes the burden due to diabetes leading to negative thoughts about diabetes in turn resulting in poor self-care behaviors[10]. Biological

underpinnings consist of one or a combination of (1) Activated immunity and inflammation mediated by cytokines; (2) Activation of HPA *via* stress; (3) Insulin resistance; (4) Disturbances of circadian rhythms; and (5) The contribution of antidepressant medications used in the treatment of depression[10] (Table 1).

Screening and diagnosis of depression

The diagnosis of diabetes, based on quantitative measurement of plasma glucose is far more refined than the diagnosis of depression; there are no biological markers for diagnosing depression. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) considers a major depressive episode as being present when at least five of nine symptoms suggestive of depression are present for 2 wk or longer; one of the nine must be a core symptom[12]. As a screening method for depression in diabetes, the Center for Epidemiologic Studies Depression Scale (CES-D) and Patient Health Questionnaire (PHQ)-9 were used most often in diabetes research[12]. Other screening tools for depression include Beck Depression Inventory, WHO well-being index and EDD[9].

Due to the non-specific nature of the symptoms and their overlap with uncontrolled hyperglycemia, the accuracy of screening tests varies between populations. Diabetes specific questionnaires are available to identify various psychological stresses[35]. There are some clinical pointers to distinguishing depression arising from diabetes and primary depression: The latter is suggested by mental disorders even before the diagnosis of diabetes, disproportionate symptoms compared to objective signs, a focus primarily on somatic symptoms and reassurance failing to relieve innocuous symptoms [36]. When these are inconclusive, screening for depression must be repeated after uncontrolled hyperglycemia is corrected[37]. However, caution must be exercised that affective symptoms such as pessimism or crying spells are not mistakenly attributed to poorly controlled diabetes[37].

For a rigorous diagnosis of depression, results of screening tests must be confirmed by a structured clinical interview such as SCID, Montgomery-Asberg Depression Rating Scale and the Composite International Diagnostic Interview[9]. These take time and require trained healthcare professionals, which limits the scope for practical application in routine clinical practice.

The reason for highlighting these aspects is to put in focus that the diagnosis of depression is subjective unlike the diagnosis of diabetes mellitus. Considering the subjective nature of diagnosing depression and the potential for false positive results, some national guidelines have not recommended population screening for depression[38]. A systematic review of screening tools for measuring depression in diabetes has shown that little data is available on their validity and reliability, with even lesser evidence for their being culturally appropriate[39]. In general, screening for major depressive disorders is based on screening instruments which do not generally consider the conceptual basis of emotional models, although efforts are being made to improve it[40]. Apart from the risk of false positive diagnosis of depression by assessing subjective methods, the outcomes of different methods of psychotherapy are not clear. The latter is being addressed by an ongoing trial: cRCT PSYCHOnline-THERAPY[41].

A Joint Position Statement of the American Diabetes Association, the American Association of Diabetes Educators, and the Academy of Nutrition and Dietetics, The American Diabetes Association recommends screening for depression in subjects with diabetes mellitus[42]. Others are advised an annual screen during major disease and life transitions.

Intervention strategies

In general, depression associated with diabetes can be managed by one or more of the following methods: Antidepressant drugs, psychological interventions such as cognitive-behavioral therapy, mindfulness-based cognitive therapy and stepped care[12].

Interestingly, many interventions that are useful in preventing and treating diabetes are also effective in depression. Physical exercise, including running helps in managing depression and other negative psychological conditions, although no quantitative measures are available to prescribe the quantum of exercise for its beneficial effects[43]. Insomnia, which often occurs with depression, is a well-known modifiable risk factor for the development of obesity and diabetes mellitus[44]. Cognitive behavior therapy for insomnia (CBT-I) is effective in improving insomnia associated with depression. CBT-I seeks to replace wrong beliefs of sleep, to help them prevent associating with stimulating activities, to limit time in bed for matching perceived sleep duration, sleep hygiene and relaxation techniques[45]. To ensure access to physical exercise and help in relaxation and ensuring adequate sleep, aspects of built environment must be considered[46].

One must recognize that guidelines for the management of depression are currently inadequately planned, reported and measured[47]. Therefore, shared decision making with the patient[48], using digital medical interview assistant systems at the primary care level could be employed to improve compliance and thereby management outcomes[49].

Although antidepressant medications are effective in the treatment of depression associated with diabetes, attention must be paid to their potential role in leading to obesity and insulin resistance[31]. Selective serotonin reuptake inhibitor agents (SRRI) are the drugs of choice, while considering the potential risk of hypoglycemia; should tricyclic antidepressants be required, one must carefully monitor glycemic control[50]. Along with antipsychotic medicines anti-depressants lead to weight gain which

Table 1 Links between type 2 diabetes and depression

Links	
Genetic	SNPs predisposing to diabetes predict symptoms associated with depression[27]
Common soil hypothesis[10]	Chronic inflammation
	Sedentary habits leading to obesity
	Activation of hypothalamic-pituitary-adrenal axis
	Disordered circadian rhythm
	Vascular dysfunction
Coincidental occurrence of both[10]	Sharing common environmental and lifestyle factors

ranges from 0.43 to 4.45 kg, with its attendant adverse metabolic effects through weight gain itself or its effects on the pancreatic beta cells. Dyslipidemia may result from the use of valproic acid derivatives, carbamazepine, mirtazapine. SSRIs can lead to dyslipidemia. Clozapine, olanzapine, valproic acid derivates and tricyclic antidepressants are known to induce insulin resistance and diabetes mellitus[51]. Newer agents such as bupropion and agomelatine, although promising, need more evidence for their therapeutic utility. Pharmacological agents used along with psychotherapy could prove to be more effective than either alone.

In a meta-analysis of 14 randomized clinical trials involving 1724 subjects, van der Feltz-Cornelis *et al* [52] concluded that treatment can improve clinical outcomes, although the combined effect of all interventions is moderate on the clinical impact[52]. When combined with diabetes self-management, psychotherapeutic interventions have a moderate clinical impact. Employing collaborative care *via* stepped care intervention is possible at the primary care level. Drug therapy and collaborative care successfully reduced depressive symptoms but did not have a significant effect on glycemic control[52].

Constraints of treating depression in diabetes

While the association between diabetes and depression, as well as the need for managing both together are recognized, implementation faces many barriers. As alluded to earlier, the diagnosis of depression is a work in progress; the burden of diabetes is so overwhelming that the identification of depression gets diluted due to lack of both time and knowledge[52]. Considering depression and diabetes are best treated together, effective management requires an embedded integrated approach rather than treating each independently[9]. It is imperative that new treatment paradigms must be identified, developed and applied to manage the twin problems of diabetes and depression, *i.e.* to democratize the treatment processes.

INTEGRATED CARE OF DIABETES AND DEPRESSION

Primarily, studies on interventions for depression showed that integrating mental health treatment to primary care settings is possible through collaborative care[53]. The key component of the collaborative care model is care managers, who are non-physicians, often nurses or social workers. Under the supervision of a physician and a psychiatrist, they identify depression by using screening tools and further provide problem-solving therapy[53]. Although encouraging in principle, a number of practical limitations remain for its wider applicability.

Compelling evidence is building up for efficacy of collaborative care in improving both glycemic control and outcomes of depression treatment[54]. Improvement of glycemia operates through better compliance to treatment[42]. It remains to be seen if the collaborative care model can be implemented at the primary care level without the need for significant additional resources. Larger studies involving cost-effective outcomes are required to determine the feasibility of such approaches[53]. Similar conclusions were drawn in a systematic review and meta-analysis on the effect of collaborative care in subjects with depression and diabetes mellitus[55,56]. From eight studies which included 2238 patients, collaborative care improved response to treatment of depression, remission of depression and better compliance to medications (anti-depressants and anti-diabetes drugs); however, there was no significant improvement of glycemic control as assessed by glycosylated hemoglobin[56]. Collaborative care involves coordination among physicians, nurses, other specialists and professionals providing management specific to the patient using evidence-based guidelines.

INtegrating DEPrEssioN and Diabetes treatmENT study

The INtegrating DEPrEssioN and Diabetes treatmENT (INDEPENDENT) Study was carried out[57] to assess whether it would be possible to bridge the gap between the high prevalence of depression in

diabetes and lack of qualified psychiatrists. It was a collaborative care model involving care coordinator, endocrinologist/diabetologist and psychiatrist in four centers in India. It assessed whether depression, identified by PHQ-9 can be managed by care coordinators, who are not professional psychiatrists, but were trained to identify and help solve issues of treatment compliance and coping with stresses. Coordination was carried out with the family and with the other members of the healthcare team of the primary physician, endocrinologists/diabetologists and psychiatrists. This follow up study was carried out in four sites in India: Madras Diabetes Research Foundation, Dr. Mohan's Diabetes Specialties Centre, Chennai, Department of Endocrinology, AIIMS, New Delhi, Endocrine and Diabetes Centre, Visakhapatnam, Diacon Hospital, Bangalore. The primary aim was to see whether there would be an improvement in depressive symptoms and metabolic parameters and whether they would be sustained for 12-mo after active intervention[58]. In the parallel, open-label, pragmatic randomized clinical trial (n:196 intervention group; n:208 controls), those who were in the intervention group were given 12 mo of support for self-management by nonphysician care-coordinators, decision support based on electronic medical records, under the periodic reviews by endocrinologists/diabetologists and psychiatrists. After a further 12-mo period of follow up without intervention, the outcomes were assessed. Control subjects received usual care for 24 mo[58]. Collaborative care intervention led to improvements in composite measure of depressive symptoms and indices of cardiometabolic health at the end of 24 mo [45].

Treatment aspects were obtained from published literature which were further adapted to local conditions by qualitative interviews involving patients with diabetes and their significant others[59]. To assess adaptations that were made to behavioral intervention made by care coordinators, and how patients responded to them, a purposive sample of patients (n:62) and care coordinators (n:3) were recruited from two clinics. Patients were interviewed about their experiences in the care model and care coordinators were interviewed about their experiences in implementation of interventions[46]. The adaptations sought and made were categorized by how they helped to improve implementation in the local context. They in turn served to help improve communication of health and to enhance engagement by the patients[59].

The use of care coordinators in managing depression among subjects with type 2 diabetes has shown promising results a year following active interventions. Further follow up and replication in other settings should be carried out to assess the generalizability of the findings from INDEPENDENT study. Recently, anxiety was shown to respond favorably to interventions in the INDEPENDENT study[60].

CRITICAL SUMMARY OF TYPE 2 DIABETES AND DEPRESSION

Judging from the number of publications, one could draw an erroneous opinion that the relationship between depression in type 2 diabetes is fully established, that effective treatment options are available and that the only constraint is to scale up intervention strategies to manage depression and type 2 diabetes. At the outset there is an asymmetry in the diagnoses of both conditions: Whereas diabetes is identified by objective criteria involving measurement of biomarkers, the diagnosis of depression is based on subjective criteria. The results from self-administered questionnaires and expert face to face interviews often diverge, as do different forms of questionnaires. The sensitivity and specificity of questionnaires need to be refined by including the cultural contexts of different populations. Therefore, there is a spectrum of conditions of what is referred to as depression associated with type 2 diabetes, from diabetes distress to subclinical depression, stretching to full blown depression. Interventions improve the outcomes of depression and of diabetes distress; however, treatment of depression improves depressive symptoms, without significant improvement of metabolic control. In contrast, treatment of diabetes distress results in improved glycemic control. Furthermore, the measures to manage them are varied and there are no accepted standard methods, rendering comparisons difficult. Therefore, despite epidemiological and mechanistic evidence for the co-existence of depression and type 2 diabetes mellitus, further refinements are necessary to define and measure the outcome of different treatment modalities of depression. However, most studies report improvement of depressive symptoms with interventions despite equivocal or no improvement of glycemic control. Therefore, it is worthwhile to identify depression in type 2 diabetes mellitus, and provide treatment by psychological and pharmacological measures. Although depression has been shown to respond to treatment, care must be taken in the choice of anti-depressant medications, some of which can worsen insulin sensitivity leading to adverse metabolic consequences. There is a lack of qualified mental care specialists to deal with the burgeoning burden of diabetes and depression. The employment of trained clinical care coordinators is a worthwhile attempt to improve access to subjects with type 2 diabetes having coexistent depressive symptoms. Preliminary results suggest the efficacy of such interventions. Further studies must be carried out to scale up across different cultural, ethnic and geographic populations.

CONCLUSION

It is established that diabetes and depression often coexist and must be managed together rather than individually. Interventions must be made across a spectrum to prevent, identify and manage depression when it occurs. Proof of principle studies have shown that they are feasible. It is necessary to scale-up such studies to assess their feasibility for wide-spread use in terms of applicability, efficacy and in terms of cost-benefit outcomes.

Non physician trained clinical coordinators can provide self-management education and support in terms of nutrition, lifestyle, compliance to medications, monitoring of metabolic parameters and dealing with psychosocial problems. These must necessarily be adapted to the age group, culture and language of the population by making appropriate cultural changes in education[16]. Depending on the availability and applicability, online interventions can be profitably made in terms of digital medical interview assistant systems[36]. With the widespread use of electronic medical records in diabetes care, a rule-based system can be incorporated so that standardized collection of data can be streamlined[61]. As the next logical step, the data can be analyzed and machine-learning methods can be devised to improve the communication, care and outcomes of diabetes and its associated morbidities including depression[62].

FOOTNOTES

Author contributions: Sridhar GR did the literature search and wrote the manuscript.

Conflict-of-interest statement: Gumpeny R Sridhar has no conflicts of interest. He has not received fees for serving as a speaker, nor has he received research funding. He does not own stocks and/or shares.

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 213-223

DOI: 10.4239/wjd.v13.i3.213

ISSN 1948-9358 (online)

MINIREVIEWS

Humanin and diabetes mellitus: A review of *in vitro* and *in vivo* studies

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A, A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Darenskaya MA, Lauro D, Liu D, Ma JH, Xia YK

Received: August 30, 2021
Peer-review started: August 30, 2021
First decision: October 3, 2021
Revised: October 24, 2021
Accepted: February 23, 2022
Article in press: February 23, 2022
Published online: March 15, 2022

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Abstract

Humanin (HN) is a 24-amino acid mitochondrial-derived polypeptide with cyto-protective and anti-apoptotic effects that regulates the mitochondrial functions under stress conditions. Accumulating evidence suggests the role of HN against age-related diseases, such as Alzheimer's disease. The decline in insulin action is a metabolic feature of aging and thus, type 2 diabetes mellitus is considered an age-related disease, as well. It has been suggested that HN increases insulin sensitivity, improves the survival of pancreatic beta cells, and delays the onset of diabetes, actions that could be deployed in the treatment of diabetes. The aim of this review is to present the *in vitro* and *in vivo* studies that examined the role of HN in insulin resistance and diabetes and to discuss its newly emerging role as a therapeutic option against those conditions.

Key Words: Diabetes mellitus; Insulin resistance; Humanin; Aging; Apoptosis; Oxidative stress

Core Tip: Humanin (HN) exerts cyto-protective and anti-apoptotic effects. Type 2 diabetes mellitus (T2DM) is considered an age-related disease. Beyond the role of HN against age-related diseases, it increases insulin sensitivity, improves the survival of pancreatic beta cells, and delays the onset of diabetes. Altered HN levels could serve as a potential biomarker in prediabetes and T2DM, since they seem to be an effect or a response to the increased reactive oxygen species production, oxidative stress, and reduced mitochondrial DNA copy number-A major and important question is whether HN could be used as a potential therapeutic option for diabetes.

Citation: Boutari C, Pappas PD, Theodoridis TD, Vavilis D. Humanin and diabetes mellitus: A review of *in vitro* and *in vivo* studies. *World J Diabetes* 2022; 13(3): 213-223
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/213.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.213

INTRODUCTION

Twenty years ago, three independent laboratories discovered humanin (HN) (MTRNR2), the first mitochondrial small open reading frame (sORF)-encoded microprotein found to have biological activity. The Hashimoto laboratory discovered HN while searching for survival factors in the unaffected brain section of an Alzheimer's patient[1]. The investigators revealed a cDNA fragment that mapped back to the mitochondrial 16S rRNA. This microprotein was named humanin because it displayed protection against Alzheimer's disease (AD)-related neurotoxicity, an action that the original authors though potentially could retrieve the "humanity" of patients suffering from dementia. Second, Ikonen *et al*[2] found that HN bound insulin like growth factor binding protein 3 (IGFBP3) using a yeast two-hybrid screening system and intensified the protective effects of IGFBP3 against amyloid-β (Aβ) toxicity. Also, Guo *et al*[3] showed that HN can bind and suppress the apoptotic protein BAX and, subsequently, alleviate cell apoptosis.

Physiologically, HN is produced by tissues in several organs, including kidney, skeletal muscles, brain, heart, and liver[4-6]. Subsequently, it is secreted into the blood circulation and transported to various target cells, protecting in parallel cells against several diseases strongly associated with oxidative stress, mitochondrial dysfunction, and cytotoxicity[7]. Beyond the cytoprotection HN possesses a key role in cell metabolism and mediates the production and secretion of endocrine/paracrine/autocrine protective stress response factors[8]. Additionally, it plays a role in age-related diseases and several metabolic disorders (*e.g.*, cardiovascular diseases [CVD], memory loss, stroke, diabetes type 2 [T2DM]).

Diabetes is a chronic disease that occurs either due to autoimmune destruction of the pancreatic beta cells, leading to absolute insulin deficiency (T1DM) or due to progressive attenuation of insulin secretion on a background of insulin resistance resulting in relative insulin deficiency (T2DM). The number of people with diabetes rose from 108 million in 1980 to 422 million in 2014. Prevalence has been increasing faster in low- and middle-income countries than in high-income countries. The rising burden of T2DM is a major concern in health care worldwide. In 2017 6.28% of the worldwide population was affected by T2DM. It is disconcerting that the burden of the disease is rising globally, and at a more rapid rate in developed regions such as western Europe[9]. As for the T1DM, its incidence is estimated 15 per 100000 people and the global prevalence 9.5%[10]. Since diabetes and its complications affect individuals' functional capacities and quality of life leading to significant morbidity and premature mortality, effective agents are required for its treatment.

STRUCTURE OF HUMANIN PEPTIDE

HN is encoded by a sORF within the gene for the 16S ribosomal subunit in the mitochondrial genome [11]. HN has a positively charged N-terminal (Met-Ala-Pro-Arg), central hydrophobic region (Gly-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Ser-Glu-Ile-Asp-Leu), and negatively charged C-terminal (Pro-Val-Lys-Arg-Arg-Ala)[1]. Last three amino acid residues in the C-terminal are considered as dispensable because both 21 and 24-amino acid long peptides have indistinguishable intracellular and extracellular effects [12]. Thirteen nuclear-encoded HN isoforms have been identified. HN-like ORF has been named MTRNR2L1 to MTRNR2L13 after the original humanin MTRNR2 gene in the mitochondrial genome. MTRNR2L1—MTRNR2L10 are expressed in most human tissues, with MTRNR2 being expressed in higher proportion in comparison to the other isoforms. Molecular manipulations of HN at key amino acids lead to changes in chemical characteristics. Additionally, single amino acid substitutions can lead to significant modifications to its biological functions and potency[13].

MECHANISMS OF ACTION

HN exerts its functions after connecting to either intracellular molecules or cell membrane receptors (Figure 1). Immediately after HN's receptor binding, extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation increases[14]. Once ERK1/2 is phosphorylated, it separates from its anchoring proteins, and transfers to other subcellular compartments. ERK1/2, a member of the mitogen-activated protein kinase pathway, participates in several essential cellular processes such as cell proliferation, survival, differentiation, mobility, and apoptosis[15,16]. HN behaves as a link to two different types of receptors: the seven-transmembrane G protein-coupled receptor formyl peptide receptor-like 1 (FPRL1) which plays a role in the cytoprotective properties of HN and a trimeric receptor, consisting of ciliary neurotrophic factor receptor (CNTFR), the cytokine receptor WSX-1 and the transmembrane glycoprotein 130 (GP130) (CNTFR/WSX-1/GP130) which is essential for HN activity and its neuroprotective effects[17]. As regards GP130, it is a transmembrane protein that acts as the signal transduction unit of the IL-6 receptor family[18]. Dimerization of GP130 receptors provokes the stimulation of janus kinases (JAK1 and JAK2), then subsequently provokes signal transducer and activator of transcription 3 (STAT3) and STAT1[19]. The dimerized STATs move to the nucleus and control transcription. The second signaling pathway directed by GP130 recruits SHP-2. SHP-2 is phosphorylated by JAK and interacts with growth-factor receptor bound protein 2 (Grb2), which allows the activation of mitogen-activated protein kinase (MAPK)[19].

HN is regulated by insulin-growth factor 1 (IGF-1) and growth hormone (GH). HN and IGF-1 Levels decrease with age[20]. It has also been suggested that GH inhibits HN levels *via* IGF-1. Treatment with GH or IGF-1 reduces circulating HN levels in both mice and human subjects[21]. To date, HN has been suggested to play a role in various diseases like T2DM[22,23], CVD[4,5], memory loss[24], amyotrophic lateral sclerosis (ALS)[25], stroke[26], and inflammation[12,27]. The main mechanisms that dominate and link these age-related diseases are oxidative stress[28] and mitochondrial dysfunction[29]. Mitochondria are principal sources of reactive oxygen species (ROS) which can cause oxidative stress and injure of the lipids, proteins, and DNA of the cells. This can afflict mitochondrial function, and, subsequently, enhanced ROS production occurs[29]. These circumstances contribute to cellular damage, apoptosis, and cellular ageing, causing ageing and age-related diseases such as Parkinson's disease[30], Alzheimer's disease[31], atherosclerosis[32], heart failure[33], myocardial infarction[34], chronic inflammation[35], kidney disease[36], stroke[37], cancers[38], and many kinds of metabolic disorders[39,40].

Especially concerning diabetes, HN provides protection against apoptosis by binding pro-apoptotic Bax, inhibiting its mitochondrial localization, and lessening Bax-mediated apoptosis activation[3], acting either directly on Bax or through the FPRL-1 receptor[17]. As for its neuroprotective action, which has also a place in the neuroendocrine beta cells protection, it involves HN binding to a complex involving CNTFR/WSX-1/GP130[17] and activation of tyrosine kinases and STAT-3 phosphorylation[41]. Moreover, an important mechanism of cell protection may be *via* interfering with Jun N-terminal kinase (JNK) activity[42]. Important is also the interaction between HN and insulin-like growth factor binding protein-3 (IGFBP-3) which prevents the activation of caspases[2]. Furthermore, an alteration at position [Gly14]-HN (S14G, HNG) seems to induce neurosurvival activity and a substitution of phenylalanine in the 6th position with alanine (F6A, F6AHN) changes the binding of HN to IGFBP-3 and enhances its main effect on glucose metabolism and insulin sensitivity[5].

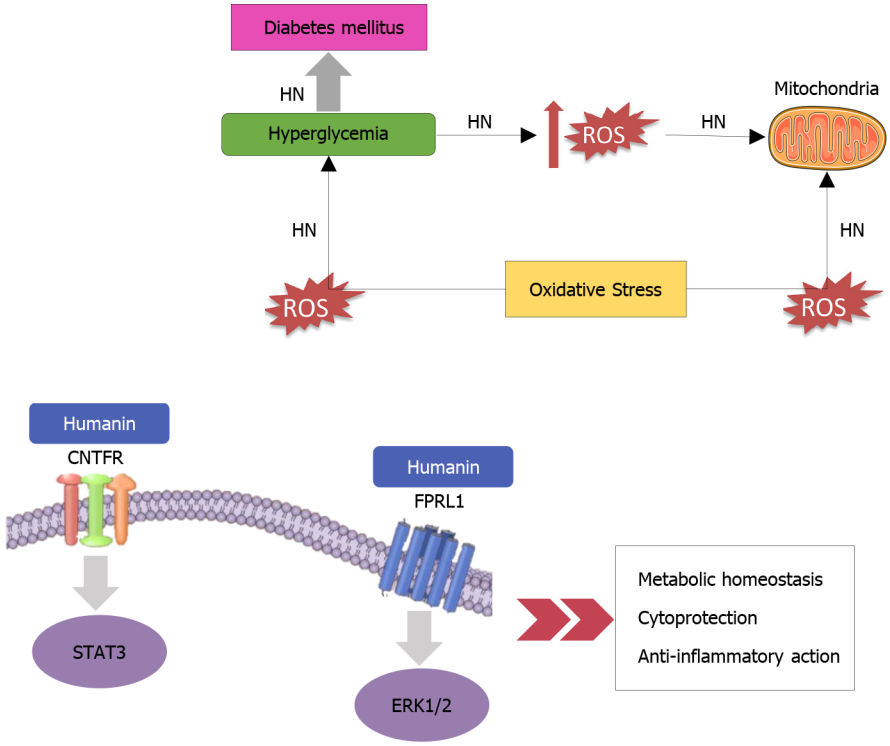
ROLE OF HUMANIN IN THE PATHOGENESIS OF TYPE 1 DIABETES

The role of HN in T1DM has been scarcely investigated. T1DM is characterized by the loss of pancreatic beta cells which results in insulin deficiency. The beta cells destruction, the dominant event in the pathogenesis of T1DM, occurs as a result of the IL-1, TNF-a, and IFN- γ actions which are originated from T cells and macrophages. Since HN is identified as a survival factor[43], it seems to serve also as a survival factor for neuroendocrine beta cells by decreasing cytokine-induced apoptosis and subsequently, improves glucose tolerance and onset of diabetes as it has been demonstrated in NOD mice *in vivo*[44]. Yet, no studies juxtaposing the HN levels in T1DM and T2DM have been published thus far.

ROLE OF HUMANIN IN THE PATHOGENESIS OF TYPE 2 DIABETES

T2DM is one of the most common metabolic diseases. This metabolic disorder and its comorbidities and complications, such as CVD, stroke, chronic kidney disease (CKD), and cancer, are global health problems which, noticeably, diminish quality of life and life expectancy[45-48].

Mitochondrial dysfunction and oxidative stress are involved in the pathogenesis of diabetes. Mitochondria are principal elements for the maintenance of metabolic health and cellular energy homeostasis. Mitochondrial dysfunction causes glycaemic dysregulation and metabolic derangement [49]. It causes inefficiency in the electron transport chain and beta-oxidation, thus trigging insulin



DOI: 10.4239/wjd.v13.i3.213

Figure 1 Mechanisms of action of Humanin in diabetes mellitus. CNTFR: Ciliary Neurotrophic Factor Receptor; ERK1/2: Extracellular signal-regulated protein kinases 1 and 2; FPRL1: Formyl peptide receptor-like 1; HN: Humanin; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3.

resistance[50]. Furthermore, hyperglycemia provokes ROS generation which, in turn, causes oxidative stress in several tissues, cellular lipids, proteins, and DNA, and subsequently, provokes chronic inflammation[51]. The accumulation of oxidative damage leads to a decrement of mitochondrial function which can result in increased ROS production[29]. It has been suggested that mitochondrial dysfunction is implicated in diabetes-related complications impairing the kidneys, nervous system, heart and retina, and that mitochondrial dysfunction-related oxidative stress contributes to these complications[52]. Subsequently, an increase in ROS concentrations may provoke HN mobilization from various tissues to the impaired areas, where HN acts against oxidative stress, decreases ROS production, and promotes cell survival[51]. Mitochondrial derived peptides (MDPs), such as HN, have been suggested to play a critical role in reducing oxidative stress[53-55] and improving T2DM[56]. It has also been demonstrated that HN promotes mitochondrial biogenesis in pancreatic β-cells[57].

IN VITRO AND ANIMAL STUDIES

In vitro and animal studies

Considering that diseases related with ageing, named T2DM and neurodegeneration, have been suggested to be associated with mitochondrial dysfunction[58,59], it follows that the mitochondrial-derived peptide HN regulates them (Table 1). Based upon the molecular interaction between HN and IGFBP-3, that prevents the activation of caspases, and since IGFBP-3, independent of IGF-1, provokes IR both at the liver and periphery[60,61], Muzumdar *et al*[23] hypothesized that HN, besides its neuroprotective action, may regulate glucose homeostasis. Utilizing state of the art clamp technology, they investigated the role and the mechanism of action of central and peripheral HN in glucose metabolism. They finally demonstrated that infusion of HN improves both hepatic and peripheral insulin sensitivity and that hypothalamic STAT-3 activation is essential for the insulin-sensitizing action of HN. Moreover, treatment with a highly potent HN analog significantly lowered blood glucose in Zucker diabetic fatty rats. As for the levels of HN in tissues like hypothalamus, skeletal muscle, and cortex, they reduced with age in rodents, and its' circulating levels were also diminished with age in humans and mice.

A year later, a group from California[44] investigated whether HN could improve the survival of beta cells and delay or even treat diabetes in NOD mice. HN prevented apoptosis induced by serum starvation in NIT-1 cells and decreased cytokine exposure-related apoptosis (caused by interleukin [IL]-1β, tumor necrosis factor [TNF]α, and interferon[IFN]γ). STAT3 is considered as a principal survival

Table 1 *In vivo* and *in vitro* studies on humanin and diabetes mellitus

Ref.	Study model	HN dose	Treatment duration	Results
In vitro studies				
Rochette *et al*[51], 2014 (HN)	NIT-1 cells	1-10000 nmol/L	24 h	Reduced apoptosis caused by serum starvation in NIT-1 cells and decreased cytokine-induced apoptosis
Hunter and Jones [19], 2015 (HNGF6A)	Isolated islets and cultured murine β cell line	50 ng/ml	15-120 min	Enhanced glucose-stimulated insulin secretion
Qin *et al*[57], 2018 (HNG)	HUVECs	1 μM	3 h	Inhibited cell death, nucleus pyknosis and deformation. Diminished the expression of cleaved PARP (which reflects the level of apoptosis as well as ROS) Decreased the level of bax (a pro-apoptotic protein). Increased bcl-2 (an anti-apoptotic agent)
Miller *et al*[11], 2020 (HNG)	HEK293 and SH-SY5Y cells	100 μM	30 min	Is a major GP130 agonist which acts through the GP130/IL6ST receptor complex and activates AKT, ERK1/2, and STAT3
Wang *et al*[50], 2010 (HN)	Pancreatic MIN6 β-cells	25, 50 and 100 μM	24h or48 h	Increased the expression of PGC-1α.Promoted mitochondrial biogenesis. Caused the phosphorylation of AMPK, improved mitochondrial respiration and stimulated ATP generation
Kim *et al*[60], 2007 (HN)	HUVECs	200 μM	24 h	Promoted the expression of KLF2.Reduced the expression of VCAM-1 and E-selectin; Impeded the secretion of TNF-α and IL-1β
In vivo studies				
Animals				
Hunter and Jones [19], 2015 (HNGF6A)	Sprague−Dawley rat	0.07 mg/kg/h	2-30 min	Improved insulin sensitivity and help in decreasing blood glucose level
Gong *et al*[20], 2014 (HN)	Sprague−Dawley rat	0.375 mg/kg/h	360 min	Decreased blood glucose in Sprague−Dawley rats by STAT-3 phosphorylation
Gong *et al*[20], 2014 (HNGF6A)	Zucker diabetic fatty rat	0.05 mg/kg/h	90-240 min	Decreased blood glucose in Zucker diabetic fatty rats
Rochette *et al*[51], 2014 (HN)	NOD mice	0.7 mg/kg/day	6 wk20 wk	Decreased lymphocyte infiltration in mice pancreata; Delayed or prevented the onset of diabetes in NOD mice (when the treatment was extended up to 20 wk)
Miller *et al*[11], 2020 (HNG)	Male C57BL/6 mice	5 mg/kg/day	2 wk	Old mice, but not young mice, showed an increase in phosphorylation in AKT and ERK1/2 in the hippocampus
Humans				
Muzumdar *et al* [61], 2006	Participants attending a diabetes complications screening clinic	-	-	A significant decrease in HN was observed in the IFG group compared to control
Ha[71], 2006	Uncomplicated T1DM patients	-	-	Plasma HN levels were significantly higher in T1D men by comparison with the healthy control men
do Nascimento *et al* [72], 2013	Pregnant women with and without GDM	-	-	Serum HN levels were significantly lower in women with GDM compared to controls
Hashimoto *et al*[42], 2003	Normal, prediabetes and diabetes subjects	-	-	Serum HN concentrations are lower in T2DM and correlate with HbA1c

HUVECs: Human umbilical vein endothelial cells; PARP: Poly ADP-ribose polymerase; ROS: Reactive oxygen species; ERK1/2: Extracellular signal-regulated kinase 1/2; STAT3: Signal transducer and activator of transcription 3; PGC-1α: PPAR-γ coactivator-1α; KLF2: Krüppel-like factor 2; VCAM-1: Vascular cell adhesion molecule 1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IFG: Impaired fasting glucose; T1DM: Type 1 diabetes mellitus; GDM: Gestational diabetes mellitus.

signaling protein in beta cells, regulating the pro-survival effects of various growth factors and cytokines. HN activated STAT3 and ERK over a 24-hour time course. Interestingly, HN improved glucose tolerance in NOD mice and after 6 wk of treatment decreased lymphocyte infiltration was observed in their pancreata. When the treatment was extended up to 20 wk the investigators noted that HN delayed or prevented the onset of diabetes in NOD mice.

A few years later, the group we mentioned first[23] hypothesized that HNGF6A, a potent non-IGFBP-3 binding HN analog, may affect acutely and independently insulin secretion, since insulin concen-

trations were not reduced along with hypoglycemia caused by HNGF6A in Sprague Dawley rats[22]. Sprague Dawley rats that received HNGF6A presented higher insulin levels during hyperglycemic clamps compared to controls. Similarly, *in vitro*, HNGF6A enhanced glucose-stimulated insulin secretion in isolated islets and cultured murine β cell line. This effect was dose dependent, combined with ATP production in the β cell, related to the KATP-channel-independent augmentation phase of insulin release[62], and associated with amplified glucose metabolism. These potent effects on insulin secretion in combination with the effects on insulin action suggested a role of HN in the treatment of T2DM.

The protective effects of [Gly14]-Humanin (HNG) against high glucose-induced apoptosis were investigated in human umbilical vein endothelial cells (HUVECs). Pretreatment of HUVECs with HNG inhibited cell death, nucleus pyknosis and deformation[63]. Also, HNG diminished the expression of cleaved poly ADP-ribose polymerase (PARP) which reflects the level of apoptosis as well as reactive oxygen species (ROS). Regarding the level of bax, which is a pro-apoptotic protein, it decreased after pretreatment with HNG, while bcl-2, which exerts anti-apoptotic effects, it increased.

Another group identified a different sORF within the mitochondrial 12S rRNA encoding a 16-amino-acid peptide named MOTS-c (mitochondrial open reading frame of the 12S rRNA type-c) which also regulates insulin sensitivity and metabolic homeostasis[56]. Particularly, MOTS-c treatment in mice protected against age-dependent and high-fat-diet-induced insulin resistance and diet-induced obesity as well. Finally, they suggested that MDPs, like MOTS-c and HN, with such systemic effects may be useful in ameliorating the abnormal metabolism associated with aging in humans and regulating biological processes like weight and metabolic homeostasis.

Kim and his colleagues from California tried to elucidate the signaling pathways underlying HN'S cytoprotective roles *in vitro* and *in vivo*[14]. Utilizing multiple models, they showed that HN is a major GP130 agonist which acts through the GP130/IL6ST receptor complex and activates AKT, ERK1/2, and STAT3. PI3K, MEK, and JAK were suggested to be involved in the activation of those three signaling pathways, respectively.

Concerning the effects of HN on mitochondrial biogenesis in pancreatic β-cells, HN treatment in MIN6 β-cells increased the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α)[57] which promotes mitochondrial biogenesis by activating the expression of nuclear respiratory factor 1 (NRF-1) and mtDNA transcription factor A (TFAM)[64]. Also, HN treatment promoted mitochondrial biogenesis by increasing mitochondrial mass, elevating mitochondrial DNA (mtDNA)/nDNA ratio (reduced mtDNA copy number plays a key role in insulin resistance[65]), and increasing cytochrome B expression. Finally, HN treatment resulted in the phosphorylation of AMPK, which was involved in the induction of PGC-1α, NRF-1, and TFAM and improved mitochondrial respiration and stimulated ATP generation leading to a possible functional gain of the mitochondria.

In HUVECs also, HN displayed protective action against high-glucose-induced endothelial dysfunction and macrovascular complications[66]. HN treatment promoted the expression of Krüppel-like factor 2 (KLF2), a principal transcriptional regulator of endothelial function, by activating ERK5. In addition, HN significantly reduced the expression of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, which regulate the adhesion of circulating leukocytes to the endothelium, a principal procedure in the initiation of atherosclerosis. Furthermore, HN impeded the secretion of pro-inflammatory cytokines, such as TNF-α and IL-1β.

HUMAN SUBJECTS RESEARCH AND CLINICAL TRIALS

Human subjects research and clinical trials

The first attempt to measure HN levels in a clinical population with impaired fasting glucose (IFG) was made in participants attending a diabetes complications screening clinic (DiabHealth)[67,68]. Previous clinical studies reported noticeably increased HN levels in patients with mitochondrial encephalomyopathy, lactic acidosis, and stroke−like episodes (MELAS) and chronic progressive external ophthalmoplegia (CPEO), which are associated with excess oxidative stress[69,70]. However, a significant reduction (P = 0.0001) in HN was reported in the IFG group (n = 23; 204.84 ± 92.87 pg mL^{-1}) compared to control (n = 58; 124.3 ± 83.91 pg mL^{-1}) in accord with an adaptive cellular response by HN to a slight raise in fasting blood glucose level (BGL). As we described above, HN protects neuroendocrine β-cells [44] and increases glucose tolerance and insulin sensitivity[20,44]. Moreover, it is considered to interact with hydrogen peroxide and α-actinin-4 which rise during oxidative stress and IFG[71-73] and binds extracellularly with the CNTFR/WSX−1/GP130 receptor[69,74,75]. Interestingly, mild to moderate levels of ROS result in positive adaptive mechanisms of the mitochondria[76]. All these mechanisms, which benefit cell function and survival, lead to a reduction in HN levels, indicating a protective role of HN. However, with disease progression to T2DM and further oxidative stress, mitochondria may upregulate HN levels as observed in studies of Alzheimer's disease and in those of MELAS and CPEO.

These conditions are related to extensive oxidative stress which is also a key feature of DM. Particularly, hyperglycemia causes extended free radical activity and mitochondrial dysfunction which induce oxidative stress and release more ROS[76]. The advanced diseases MELAS and CPEO are

associated with increased plasma HN levels. HN has a protective role and is upregulated with disease progression. On the contrary, the minor elevations of blood glucose levels are combined with a decrease in HN concentrations which supports the protective role of HN when levels are expected to decrease as a result of stimulation of oxidative stress-associated agents that are inhibited by HN. However, with disease progression to T2DM and further oxidative stress, mitochondria increase HN levels, as reported in MELAS and CPEO.

A few years earlier, another group from Toronto suggested that plasma HN levels were significantly higher in T1D men by comparison with the healthy control men ($P < 0.0001$)[77].

At the end of 2018 Ma *et al*[78] evaluated HN concentrations in pregnant women with and without gestational diabetes mellitus (GDM) aiming to define the role of HN in the development of GDM. 157 women were enrolled in the study. Serum HN levels were significantly lower in women with GDM compared to controls. Like Lee *et al*[21], who found that HN was regulated by IGF-1 in mice and humans, they suggested that the IGF axis influenced the HN levels and affected its normal function in GDM. By performing logistic regression analysis, they also showed that low HN levels were the independent risk factor of GDM and, therefore, might be a predictor for the GDM diagnosis. Additionally, HN levels were significantly negatively correlated with the body weight, body mass index (BMI) and homeostatic model assessment for insulin resistance (HOMA-IR).

The most recent study which attempted to evaluate MDP levels in normal, prediabetes and diabetes subjects enrolled 225 participants[49]. The investigators found that serum HN concentrations are lower in T2DM ($P < 0.0001$) and correlate with HbA1c. Interestingly, HN levels decreased by 62% in the prediabetes group, 66% in diabetes subjects with good control and 77% in uncontrolled diabetes patients compared to participants without diabetes. Also, this study confirmed that there are no significant differences in HN levels between healthy men and women and the levels of HN were not affected by the different anti-diabetic treatment (insulin, metformin, other hypoglycemic regimens) or the duration of therapy. Furthermore, since HN was associated with adiponectin, which has been suggested to be reduced in prediabetes and T2DM[79], it can be concluded that mitochondrial dysfunction contributes to glycemic dysregulation and metabolic effects in T2DM. Adiponectin levels were positively correlated with HN. Adiponectin concentrations decrease in pre-diabetes and DM[79]. It has also been demonstrated that adiponectin knockout mice have reduced mitochondrial content combined with insulin resistance[80]. In addition adiponectin may impair mitochondrial biogenesis[81]. Therefore, the affected mitochondrial function may arise from the low adiponectin levels.

As for the changes in HN levels with ageing, Voigt *et al*[67] showed that HN decreased with age among individuals attending a diabetes complications screening clinic suggesting a protective function of HN and this observation was consistent with a previous study among human and mice[23]. On the contrary, circulating levels of HN increase in age-associated diseases such as T2DM. With disease progression and additional oxidative stress, mitochondria may increase HN levels.

Besides the initial and principal lifestyle interventions for glycemic control in DM, currently, we have various oral and injectable pharmacologic agents at our disposal including metformin, thiazolidinediones, sulfonylureas, glucagon-like peptide 1 (GLP-1) receptor agonists, dipeptyl-peptidase 4 (DPP-4) inhibitors, sodium-glucose co-transporter 2 (SGLT-2) inhibitors, and insulin[82]. These medicines can be administered in various dosages and in many combinations in each patient diagnosed with DM. However, there is still room for additional new factors that could efficiently contribute to the management of the disease. Given HN's protective properties, it may represent a novel treatment option to decrease the cellular damage caused by diabetes. Altered HN levels in diabetes could serve as a potential biomarker. Nevertheless, no clinical trials investigating the effects of HN or its analogues (e.g. HNGF6a) administration have thus far been published, albeit it would be an innovative and promising breakthrough in diabetes prevention and treatment.

CONCLUSION

In summary, HN shows cytoprotective effects in many biological processes, including oxidative stress and apoptosis. Altered HN levels could serve as a potential biomarker in prediabetes and T2DM, since they seem to be an effect or a response to the increased ROS production, oxidative stress, and reduced mtDNA copy number which all contribute to IR[83]. However, further study is needed to define the role of age and other modifiable confounding factors, like fitness level, adiposity, other metabolic comorbidities, such as CVD, stroke, inflammation. Undoubtedly, the major and important question is whether HN could be used as a potential therapeutic option for diabetes, that could even replace the current diabetes mellitus treatment strategies soon. Towards this direction, further studies are needed to identify the contribution of HN in the metabolic dysregulation of T2DM.

FOOTNOTES

Author contributions: All authors contributed equally to the writing of the manuscript and have read and approve the final manuscript.

Conflict-of-interest statement: There are no conflicts of interest to declare.

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S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 224-239

DOI: 10.4239/wjd.v13.i3.224

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Case Control Study

Functional annotation and enrichment analysis of differentially expressed serum proteins in patients with type 2 diabetes after dapagliflozin

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Belosludtseva NV, Cure E

Received: October 30, 2021
Peer-review started: October 30, 2021
First decision: December 27, 2021
Revised: January 29, 2022
Accepted: February 23, 2022
Article in press: February 23, 2022
Published online: March 15, 2022

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Abstract

BACKGROUND

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

AIM

To identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM.

METHODS

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

RESULTS

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin (ITGA2B), and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

CONCLUSION

Dapagliflozin has hypoglycemic effects and regulates the serum expressions of MPO, ITGA2B, and PCX, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

Key Words: Type 2 diabetes mellitus; Dapagliflozin; Non-standard quantitative proteomics; Myeloperoxidase; Alpha II B integrin; Podocalyxin

Core Tip: This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with type 2 diabetes mellitus. Changes in blood indexes were examined in 20 patients treated with dapagliflozin for 3 mo. Quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. Dapagliflozin has hypoglycemic effects and regulates the serum expressions of myeloperoxidase, alpha II B integrin, and podocalyxin, possibly contributing to the effects of dapagliflozin on oxidative stress, insulin resistance, and lipid metabolism.

Citation: Zhao YX, Borjigin S, Yan ZL. Functional annotation and enrichment analysis of differentially expressed serum proteins in patients with type 2 diabetes after dapagliflozin. *World J Diabetes* 2022; 13(3): 224-239
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/224.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.224

INTRODUCTION

With the aging global population and the increase in the prevalence of obesity, it is expected that type 2 diabetes mellitus (T2DM) will affect more than 381.8 million people worldwide in 2035[1]. In the United States alone, T2DM is projected to affect nearly one in three people by 2050[2]. T2DM manifests through the development of fasting and postprandial hyperglycemia, which is the primary contributor to numerous life-threatening complications and co-morbidities[3,4]. These alarming projections suggest an urgent need for the development and implementation of novel preventative and treatment strategies to fight the rise in T2DM prevalence worldwide[4]. Unfortunately, despite the best care, only 50% of patients with T2DM can control their blood glucose levels[5-7].

In recent years, the participation of the kidney in glucose metabolism and homeostasis attracted much attention, and this participation has begun to be explored in clinical studies[8]. The mechanisms mainly include the renal tubular reabsorption of glucose, largely dependent on the expression of sodium-glucose co-transporter 2 (SGLT-2) localized at the proximal small tubules S1 and S2[9]. Dapagliflozin is a selective inhibitor of SGLT-2, reducing the reabsorption of SGLT-2 receptor glucose in renal tubular epithelial cells and allowing excess glucose to be excreted in the urine[10]. Thus, the insulin sensitivity of the liver and peripheral tissues can be improved, and the hepatic glucose output can return to the normal range[10,11]. Furthermore, many investigators proposed that SGLT-2 inhibitors have renal and cardiovascular protective roles in addition to their glucose-lowering effects[12-14]. Thereby, dapagliflozin is recommended for T2DM patients[15].

Several studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection[13-15]. Powell *et al*[16] suggested that SGLT2 inhibitor alone could reduce hemoglobin A1c (HbA1c) by 0.37%-1.16%. Several randomized, double-blind, controlled trials have confirmed that dapagliflozin can significantly reduce HbA1c (by up to 1.16%) and blood glucose and that the efficacy of dapagliflozin (10 mg) in reducing HbA1c is

comparable to that of metformin sustained-release tablets (2000 mg)[17,18]. Ji *et al*[19] proposed that SGLT2 inhibitors can reduce blood glucose and hyperglycemic toxicity by reducing the stress reaction in the endoplasmic reticulum and reducing the beta-cell apoptosis caused by glycolipid toxicity, thereby improving insulin secretion. They also proposed that the damaged function of the beta-cells will also be improved[19]. Nevertheless, the mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially abundant proteins in serum of T2DM patients have not been intensively explored so far.

Proteomics techniques have attracted more and more attention since these techniques can be used to identify the expression of differential proteins in cells and tissues of patients with T2DM[20]. Label-free quantitative proteomics can replace or supplement the traditional two-way gel electrophoresis approach, and it has become an important mass spectrometry method in recent years because of its powerful protein identification ability[21]. The changes in protein abundances of different samples can be analyzed by comparing mass spectrometry frequency or mass spectrometry peak intensity[22]. Without expensive isotope labeling and with liquid chromatography-mass spectrometry analysis of peptides obtained from protein enzymatic digestion, the relative abundance of the corresponding proteins can be quantified according to the signal strength of the peptide segments[22]. In this study, using this technique, we explored the differentially abundant proteins in the serum samples of T2DM patients before and after dapagliflozin treatments and conducted functional annotation analysis of the differential proteins. In addition, the levels of some differential proteins in serum samples were validated, and the correlations between their levels and clinical indexes were analyzed.

MATERIALS AND METHODS

Patients

Forty-six patients with T2DM were enrolled at the Department of Endocrinology, Affiliated Hospital of Inner Mongolia Medical University, between January 1, 2017 and December 1, 2018. There were 26 participants in the dapagliflozin group, and 20 participants who controlled their blood glucose levels through diet and exercise alone were enrolled in the control group during the same period (these patients did not receive drugs as per their own choice but were still followed in case diet and exercise became insufficient to control their T2DM). All participants met the diagnostic criteria for T2DM according to the World Health Organization diagnostic criteria for type 2 diabetes in 2017[23] The course of T2DM was < 5 years. All participants were 25-55 years of age, and they did not have a blood relationship. All participants had complete physical examination data and other disease information. The exclusion criteria were: (1) Acute or chronic complications of T2DM; (2) Cardiovascular and cerebrovascular diseases such as hypertension or coronary heart disease; (3) Other antidiabetic, antihypertensive, or lipid-regulating drugs; (4) Ketoacidosis, genital fungal infection, or urinary tract infection; (5) Recent history of surgery and trauma; or (6) Infectious diseases, tumors, hematological diseases, severe impairment of heart, liver and kidney functions, autoimmune diseases and other endocrine and metabolic diseases.

This study was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University. All participants signed the informed consent form before the start of the study.

Study groups

For the participants in the dapagliflozin group, their HbA1c levels ranged from 7.0% to 10.0%. If the participants were taking other hypoglycemic drugs, the participants were admitted to the group after a wash-out period of 1-2 wk. The participants were instructed to adhere strictly to diet and exercise during the wash-out period. After wash-out, the participants were treated with dapagliflozin alone after clinical evaluation. The participants without the need for wash-out were treated with dapagliflozin directly after clinical evaluation. In order to avoid complications such as urinary tract infection and ketoacidosis, the participants were advised to drink more water during the study period, which was also conducive to the excretion of urinary glucose. No other drugs, such as lipid-lowering drugs, were allowed during the study. After 3 mo of treatment, the participants with HbA1c < 7.0% were considered as reaching the HbA1c target level. Six patients in the dapagliflozin group dropped out due to self-discontinuation, failure to meet the HbA1c target, or refusal to be reviewed after 3 mo. The average age of the 20 eligible patients in this group was 39.8 ± 5.1 years.

For the participants in the control group, their HbA1c levels reached the target level (HbA1c < 7.0%). These participants did not take any hypoglycemic drugs or other drugs within 3 mo before sampling, and they controlled their blood glucose levels only through diet and exercise. There were no dropouts. Their average age was 39.8 ± 6.0 years.

All participants received diet and exercise guidance. The dietary guidance referred to the balanced dietary plan recommended by the Chinese Guidelines for the Prevention and Treatment of Type 2 Diabetes (2013 edition) and suggested 1/3 structure of the energy intake ratio of three meals or 2:2:1 distribution. According to the "Guidelines for Exercise in Type 2 Diabetes Mellitus", the exercise program was mainly composed of low-intensity aerobic exercise such as walking, swimming, cycling,

etc. After a meal, the participants were required to exercise for about 30 min and 3-5 times per week. During the study, no adverse reactions such as urinary tract infection, ketoacidosis, or other adverse reactions occurred.

Data collection

Fasting venous blood was drawn from the participants in the morning. Blood samples were subjected to centrifugation. The supernatant was stored at -80 °C until analysis. Blood samples were also sent to the laboratory professionals of Affiliated Hospital of Inner Mongolia Medical University for quantification of indicators, including retinol-binding protein 4 (RBP4), fasting blood glucose (FBG), fasting C-peptide (FCP), fasting plasma insulin level (FINS), HbA1c, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol(LDL-C), triglycerides(TG), total cholesterol (TC), apolipoprotein A1 (ApoA1), ApoB100, homocysteine (HCY), non-esterified fatty acids (NEFA), C-reactive protein. These indicators were determined using Roche Cobas 8000 and Aikelai H-8160 automatic biochemical analyzers in the Laboratory Department of Affiliated Hospital of Inner Mongolia Medical University. Quality control was carried out by the professionals in the Laboratory Department. Insulin resistance indexes, including updated homeostasis model assessment-insulin resistance (HOMA2-IR), updated homeostasis model assessment-beta cell (HOMA2-B), and updated homeostasis model assessment-insulin sensitivity (HOMA2-S%), were obtained using the HOMA Calculator v2.2.3 software based on FPG, C-peptide, and islet function. The non-HDL-C value was calculated by subtracting the HDL-C value from the TC value.

Label-free proteomics

Serum samples from five patients in the dapagliflozin group before and after dapagliflozin treatments for 3 mo were selected for the exploratory proteomics study. Individual differences such as diabetes duration, blood glucose levels, and body mass index (BMI) were minimized to ensure the reliability of the results. High-abundance proteins were removed from the samples using Pierce™ Top 12 Abundant Protein Depletion Spin Columns Kit following the manufacturer's instruction, and total protein concentration was determined by a BCA kit (Pierce). The proteins were resolved by 10% SDS-PAGE and visualized with Coomassie Blue staining. Each lane was excised and cut into bands containing proteins with different molecular weights. Each gel fraction was subjected to in-gel tryptic digestion. Peptide segments were classified by high-pH reverse HPLC with Agilent 300 Extend C18 (5 μm diameter, 4.6 mm inner diameter, 250 mm long).

Digested peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS using a ThermoScientific Easy nLC-1000 in tandem with a Q-Exactive Orbitrap mass spectrometer. Each sample (5 μL) was resolved using a 60 min gradient (Buffer A: 0.1 formic acid in 2% acetonitrile; Buffer B: 0.1% formic acid in 90% acetonitrile) on a 2 cm Acclaim 100 PepMap Nanoviper C18 trapping column in tandem with a Thermo EASY-Spray column (PepMap® RSLC, C18, 3 μm, 100 A, 75 μm × 150 mm).

Secondary mass spectrometry data were retrieved using Maxquant (v1.5.2.8).

The database was SwissProt Human (20387 sequences), which included the counter-library in calculating the false positive rate (FDR) caused by randomly matching. The common contamination library was considered to eliminate contaminations. The enzyme cutting method was set to trypsin/P; the number of leakage sites was set to 2. The first-stage mother ion mass error tolerance to the first search and main search was set to 20 ppm and 5 ppm. The error tolerance of the secondary fragment ions mass was set as 0.02 Da. The alkylation of cysteine was set as fixed modification, while the oxidation of methionine and acetylation of protein N-terminus were set as alternative modifications. The FDR of protein identification and PSM identification was set as 1%.

For data-dependent analysis, full scans were acquired at a 35000 resolution range of 400-200 m/z, while a 17500 resolution was used for MS/MS scans. Only the top 15 ions with +2 and +3 charges were selected for MS/MS with 10-s dynamic exclusion to prevent continuous reanalysis of abundant peptides. Following data acquisition, raw data files were compiled for each gel lane and searched with Proteome Discoverer 1.4's SEQUEST search algorithm using the reviewed, non-redundant homo sapiens complete proteome retrieved from UniProtKB.

Bioinformatics analysis

For protein functional enrichment evaluation, Gene Ontology (GO) and pathway enrichment analyses were carried out. GO annotations (including biological process (BP), molecular function (MF), and cellular component) were performed using the InterProScan database (v.5.14–53.0 http://www.ebi.ac.uk/interpro/) and according to an existing report[16]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used for pathway enrichment analysis[16]. KAAS (v.2.0 http://www.genome.jp/kaas-bin/kaas_main) and KEGG mapper (v.2.5 http://www.kegg.jp/kegg/mapper.html) are the main tools used with the KEGG database. Prediction of subcellular localization was carried out using the wolfpsort software (v.0.2 http://www.genscript.com/psort/wolf_psort.html). Cluster memberships were visualized using the heat map drawn by the function heatmap.2 in the R package gplots. For each annotation, Fisher's exact test was applied to

compare the enrichment of the differentially abundant protein against all identified proteins, and a P value < 0.05 was considered significant.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was performed for the quantification of alpha II B integrin, myeloperoxidase (MPO), and podocalyxin (PCX) using the kits from Bio-Rad Laboratories, United States (CSB-EL0118644HU for integrin, CSB-E08721h for MPO, and CSB-E09891h for PCX). All reagents were equilibrated to room temperature (18-25 °C) for at least 30 min and prepared according to the instructions of the relevant kits. The optical density of each well was measured sequentially at 450 nm with an enzyme-labeled instrument within 5 min after the termination of the reaction.

Statistical analysis

The statistical methods of this study were reviewed by Xue-Mei Wang. The quantitative data were described using means ± SD. The paired *t*-test was used to compare the indexes obeying the normal distribution before and after treatment. Variance analysis was used to compare the indexes obeying normal distribution, and a nonparametric rank-sum test was used to compare the indexes not obeying normal distribution. Pearson correlation analysis was used for the two variables obeying normal distribution, and Spearman grade correlation analysis was used for the variables not obeying normal distribution. The chi-square test was used to analyze the categorical data. For the enrichment test, Fisher's exact test was used to show the functional classification and pathway of significant enrichment of differentially expressed proteins (P < 0.05) using bubble diagrams. Two-sided P < 0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS 22.0.

RESULTS

Dapagliflozin significantly improved the clinical manifestations in patients with T2DM

Twenty participants were treated from January 1, 2017 to December 1, 2018. Table 1 presents the characteristics of the participants in the dapagliflozin (after treatment) and control groups. Compared with the controls, the patients under dapagliflozin treatment had slightly higher FBP, lower FINS, and lower HOMA2-B, but no significant differences in HOMA2-S% and HOMA2-IR. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels. Table 2 presents the comparison before/after dapagliflozin. Compared with baseline, the participants after dapagliflozin treatment had significantly decreased BMI, waist circumference, and waist-hip ratio (P < 0.05) (Table 2). In addition, their blood glucose-related indexes, including HbA1c, FCP, FINS, FBP, and HOMA2-IR, also demonstrated significantly decreased levels (P < 0.05), while HOMA2-S% and HOMA2-B significantly increased (P < 0.05) (Table 2). Regarding the lipid metabolism-related indexes, non-HDL-C decreased (P < 0.05), while ApoA1 increased (P < 0.05) (Table 2). Moreover, RBP, HCY, and NEFA were significantly decreased levels after treatment (P < 0.05) (Table 2). Taken together, dapagliflozin substantially improved the clinical manifestation of patients with T2DM.

Identification of differentially abundant proteins by label-free proteomic

Next, we performed a label-free proteomics analysis of serum samples from five patients before and after dapagliflozin treatments. A total of 665007 sary spectra were obtained through mass spectrometry. A total of 4732 peptides were identified through spectral analysis, of which 3389 were specific peptides. We identified 534 proteins, all of which could be quantified (quantitative protein means that at least one comparison group has quantitative information). The evaluation of quantitative proteome reproducibility was performed by relative standard deviation (RSD) analysis, which showed that the biological replicates were statistically consistent (Figure 1A). The heatmap of the Pearson correlation coefficients from all quantified proteins between each pair of samples demonstrated that the linear correlation degree of the two metrics was not high (Figure 1B). Notably, 19 proteins exhibited significant differences before and after dapagliflozin treatments (P < 0.05; FC > 1.5), of which 18 were downregulated, and one was upregulated (Figure 1C).

In order to determine the characteristics of the differentially expressed proteins, we annotated the subcellular localization, Clusters of Orthologous Groups of proteins (KOG), Gene Ontology (GO), and KEGG pathway of the 19 differentially expressed proteins. The annotation of the subcellular localization showed that 31.6% of all differentially expressed proteins were localized to the cytoplasm, 26.3% to the extracellular space, 15.8% to the nucleus, 10.5% to the plasma membrane, 5.3% to the mitochondria, 5.3% to the endoplasmic reticulum, and 3.8% to the cytoskeleton (Figure 2A). In the GO analysis, the GO annotations are divided into three categories (biological process, cellular component, and molecular function), explaining the biological role of proteins from different perspectives. In the biological process classification of GO, the cellular process had the largest proportion, followed by the single-organism process and biological regulation. In the cellular composition classification, differentially expressed proteins were mostly expressed in organelles, followed by cells and extracellular regions. In the

Table 1 Analysis of clinical indexes between the dapagliflozin and control groups in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Dapagliflozin	Control	Wilcoxon W	*P* value
Age	39.80 ± 5.136	39.75 ± 5.990	397.000	0.724
Smoking history	16.55 ± 9.720	10.25 ±11.639	353.000	0.111
Height (m)	1.7670 ± 0.07255	1.7505 ± 0.090	389.000	0.569
Weight (kg)	74.950 ± 8.9176	72.525 ± 6.6718	381.500	0.440
BMI (kg/m^2)	24.3695 ± 1.82674	24.014 ± 1.6715	385.00	0.499
Waist circumference (cm)	96.350 ± 2.3402	93.525 ± 5.7502	325.000	0.021
Hip circumference (cm)	96.975 ± 2.4413	99.225 ± 6.4062	398.500	0.755
Waist-hip ratio	0.9937 ± 0.0132391	0.9494 ± 0.04815	308.000	0.006
SBP (mmHg)	124.80 ± 5.297	120.40 ± 5.423	323.500	0.018
DBP (mmHg)	78.85 ± 5.724	77.95 ± 5.969	394.500	0.671
Diabetes course (yr)	2.575 ± 1.2169	2.825 ± 1.8229	396.500	0.713
TG	1.884 ± 1.2172	0.979 ± 0.2376	1.2172203	0.002
LDL	2.8585 ± 1.0440	1.5975 ± 0.5079	1.0439867	< 0.001
HDL	1.0855 ± 0.2457	1.023 ± 0.1246	0.2457100	0.560
APOA1	1.3630 ± 0.2022	1.2625 ± 0.1882	0.2022271	0.010
APOB100	0.9425 ± 0.2651	1.0345 ± 0.1521	0.2651092	0.424
CRP	1.311 ± 0.9305	1.1635 ± 0.5462	0.9305511	0.967
HbA1c	6.7500 ± 0.6863	6.3700 ± 0.3827	0.6863327	0.032
THCY	12.030 ± 2.1451	12.4215 ± 1.2702	2.1450899	0.213
RBP4	37.150 ± 5.6033	35.800 ± 4.3842	5.6033	0.446
NEFA	0.8245 ± 0.5849	1.026 ± 0.1099	0.5849019	< 0.001
FCP	2.2770 ± 0.7903	2.231 ± 0.4151	0.7903104	0.525
TC	4.1950 ± 0.8378	3.7875 ± 0.4683	344.500	0.076
IR	2.7120 ± 1.7245	2.8440 ± .8424	358.500	0.163
FINS	8.4905 ± 4.4824	10.313 ± 3.2099	328.500	0.027
FBP	6.80 ± 1.105	6.15 ± 0.671	335.500	0.034
Non-HDL	3.05 ± 0.826	2.80 ± 0.410	364.000	0.142
HOMA2- insulin	75.6150 ± 20.339	89.460 ± 18.246	324.000	0.020
HOMA2-S%	61.6750 ± 21.1603	60.440 ± 14.789	400.000	0.787
HOMA2-IR	1.8185 ± 0.7285	1.7210 ± 0.3760	399.000	0.766

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

molecular function classification, binding molecules accounted for the largest proportion (Figure 2B). In terms of subcellular structure location, these differentially expressed proteins were mainly located in the cytoplasm, extracellular, nucleus, and plasma membrane. COG/KOG functional classification revealed that most of these differentially expressed proteins played a role in the cytoskeleton, extracellular structures, intracellular trafficking, secretion, vesicular transport, carbohydrate transport, and metabolism, as general function predictions (Figure 2C).

Table 2 Analysis of clinical indexes before and after dapagliflozin treatment in patients with type 2 diabetes mellitus (means ± SD, *n* = 20)

Parameter	Before dapagliflozin	After dapagliflozin	Wilcoxon W	*P* value
Weight (kg)	80.600 ± 10.4549	74.950 ± 8.9176	351.000	0.110
BMI	26.1875 ± 2.1889	24.3695 ± 1.8267	313.000	0.009
Waist circumference (cm)	98.300 ± 2.4570	96.350 ± 2.3402	310.50	0.007
Hip circumference (cm)	97.425 ± 3.1131	96.975 ± 2.4413	395.000	0.684
Waist-hip ratio	1.009350 ± 0.0172	0.9937 ± 0.0132	311.500	0.007
TG	2.3020 ± 1.7483	1.884 ± 1.2172	387.000	0.534
LDL	2.9310 ± 0.9873	2.8585 ± 1.0440	405.000	0.892
HDL	1.0525 ± 0.2722	1.0855 ± 0.2457	381.000	0.432
APOA1	1.2450 ± 0.2176	1.3630 ± 0.2022	334.500	0.041
APOB100	1.0345 ± 0.3400	0.9425 ± 0.2651	392.000	0.626
CRP	1.7090 ± 1.1454	1.311 ± 0.9305	349.000	0.098
HbA1c	8.0550 ± 1.0842	6.7500 ± 0.6863	277.000	< 0.001
THCY	14.6850 ± 3.0387	12.030 ± 2.1451	311.500	0.008
RBP4	43.310 ± 8.8547	37.150 ± 5.6033	315.500	0.010
NEFA	0.4525 ± 0.3246	0.8245 ± 0.5849	321.500	0.017
FCP	2.7980 ± 0.9510	2.2770 ± 0.7903	337.000	0.048
TC	4.6830 ± 1.1643	4.1950 ± 0.8378	347.500	0.091
IR	5.7385 ± 3.2238	2.7120 ± 1.7245	266.000	< 0.001
FINS	13.3500 ± 5.7057	8.4905 ± 4.4824	289.000	0.001
FBP	9.60 ± 2.437	6.80 ± 1.105	245.000	< 0.001
Non-HDL	3.70 ± 1.129	3.05 ± 0.826	337.000	0.037
HOMA2-insulin	53.4450 ± 21.1716	75.6150 ± 20.339	305.000	0.005
HOMA2-S%	44.7950 ± 16.2213	61.6750 ± 21.1603	310.500	0.007
HOMA2-IR	2.5185 ± 0.9686	1.8185 ± 0.7285	313.000	0.009

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; APOA1: Apolipoprotein A1; ApoB100: Apolipoprotein B100; CRP: C-reactive protein; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; THCY: Homocysteine; RBP4: Retinol binding protein 4; NEFA: Non-esterified fatty acids; FCP: Fasting C-peptide; TC: Total cholesterol; FINS: Fasting plasma insulin level; HOMA2-B: Homeostatic model assessment-beta cell; HOMA2-S%: Homeostatic model assessment-insulin sensitivity; HOMA2-IR: Homeostatic model assessment-insulin resistance.

Functional enrichment analysis of differentially abundant proteins

For the annotation of all identified proteins and the screening of differentially expressed proteins, the differentially expressed proteins in our comparison groups were enriched at three levels: GO classification, KEGG pathway, and protein domains. The purpose was to determine whether the differentially expressed proteins had significant enrichment trends in some functional types. GO functional classification found that the homotypic intercellular adhesion pathway, lymphocyte activation pathway, and actin cytoskeleton regulation pathway were highly enriched in the classification of cell composition (Figure 3A). The pathways such as Cortex and Cytoskeleton were enriched significantly in the KEGG pathway (Figure 3B). In the function of molecular biology, the small GTP enzyme binding pathway and the Ras GTPase binding pathway were enriched significantly (Figure 3C).

We found that these proteins, such as actin, alpha II beta integrin, MPO, and PCX, were closely related by the KEGG pathway. Therefore, we performed a protein-protein interaction analysis and identified a network among the integrin protein, MPO, and PCX (Figure 4). The integrin protein was used to modulate the production of cytoplasmic actin by participating in the PKC and the FAK pathway to function on actin filament and vinculin. On the other hand, by participating in the PKC and FAK pathway to function on Rac family proteins, the integrin protein modulates the MAPK signal through the PAK pathway to eventually manipulate gene expression. Besides, the integrin protein can also act

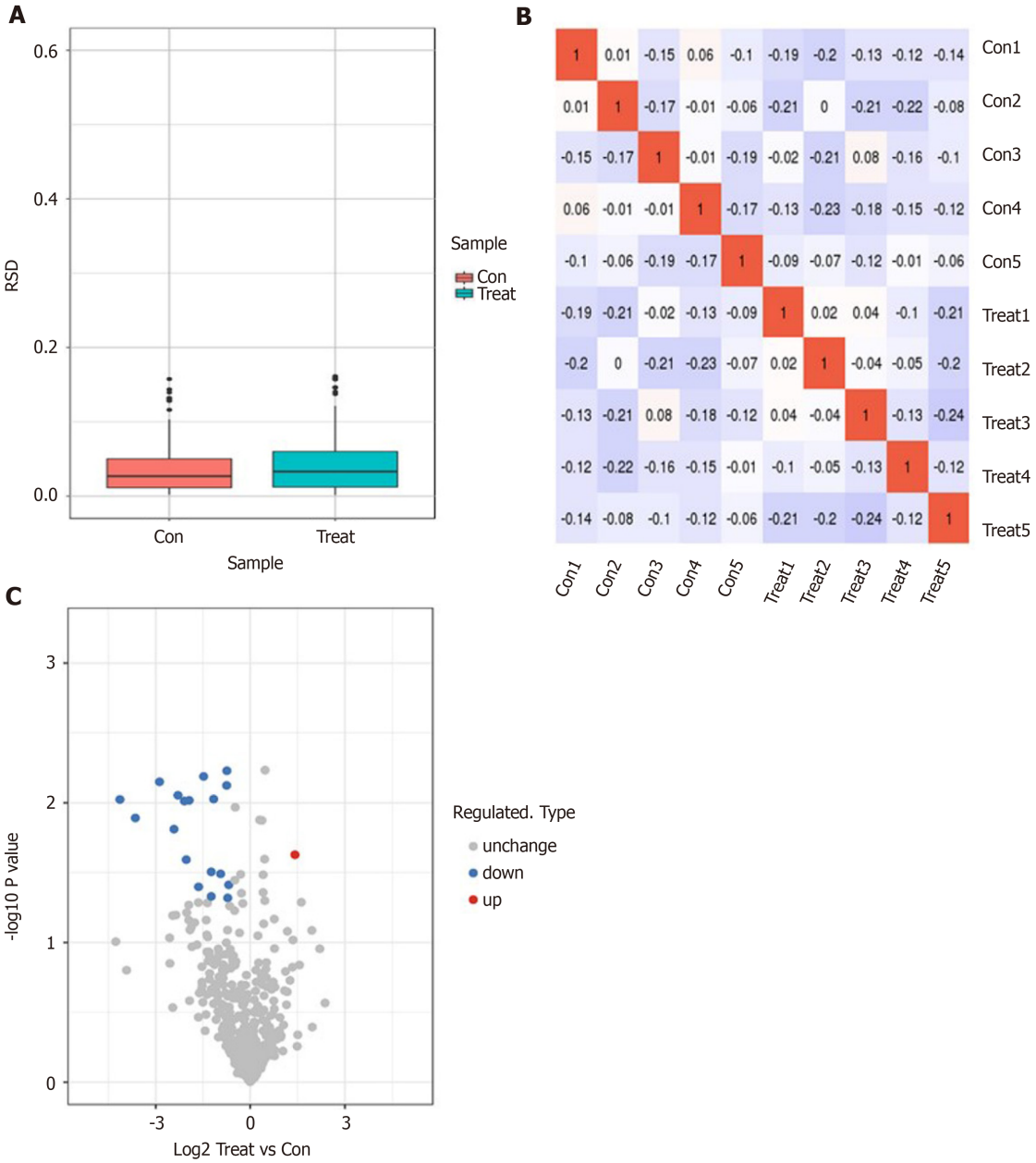

Figure 1 Identification of dapagliflozin treatment-associated differentially abundant proteins in serum samples from type 2 diabetic patients by label-free proteomics. A: Box plot of relative standard deviation (RSD) distribution of repeated samples using quantified proteins. A box plot drawn by the RSD of the quantitative protein value between replicate samples is shown. The smaller the overall RSD value is, the better the quantitative repeatability is; B: Heatmap of Pearson correlation coefficients from all quantified proteins between each pair of samples is shown; C: The volcano plot demonstrated differentially expressed proteins. The horizontal axis is the relative quantitative value of the protein after Log2 Logarithmic conversion, and the vertical axis is the value of the difference significance test *P* value after -Log10 Logarithmic conversion. The red dots in the figure indicate proteins with significantly differentially upregulated expression, and blue dots indicate proteins with significantly differentially down-regulated expression.

on the PI3K pathway through the SRC family, affecting Notch signaling pathway. The cytoplasmic actin is involved in the behavior of phagolysosome with coronin, and the MPO production is increased during the formation of autophagolysosome. PCX protein and actin are involved in regulating the actin skeleton, and the Notch signaling pathway also participates in this process (Figure 4).

Validation of three differentially expressed proteins and their correlations with clinical indexes

Based on the literature and our analysis of the biological function of candidate proteins that might be closely related to diabetes mellitus, three differentially expressed proteins (including MPO, alpha II B integrin, and PCX) were validated by ELISA. The results showed that the expression of MPO and alpha II B integrin protein in serum samples was significantly downregulated ($P < 0.05$), and the PCX protein levels were significantly upregulated ($P < 0.05$) after dapagliflozin treatment (Figure 5A). In addition, compared with the control group, the dapagliflozin group also had a significantly downregulated expression of MPO after treatment ($P < 0.05$) (Supplementary Figure 1). We also conducted a correlation

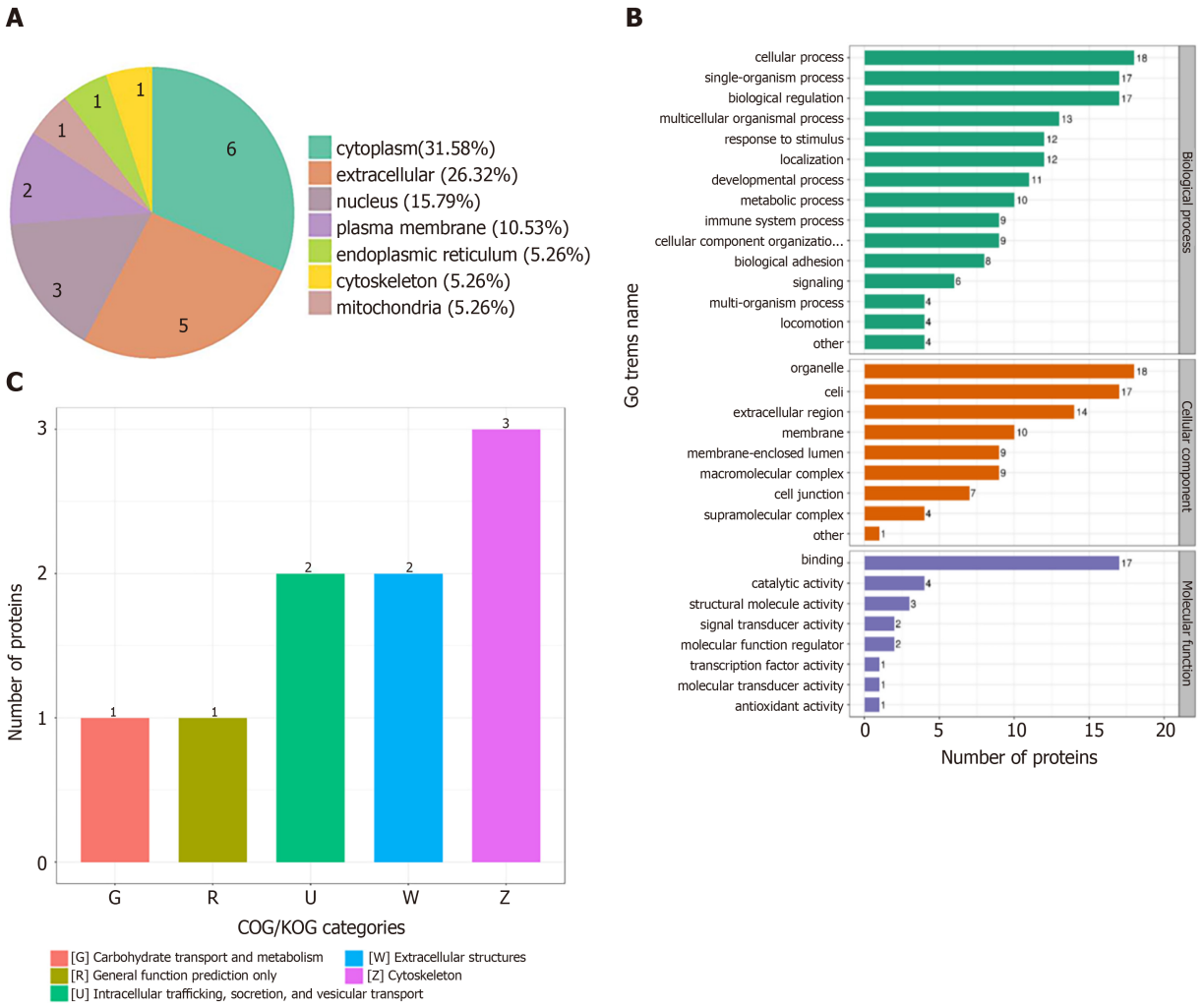

Figure 2 Annotation analysis of 19 differentially expressed proteins by subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations. A: Subcellular localization chart of differentially expressed proteins; B: Statistical distribution chart of differentially expressed proteins under each Gene Ontology category (2nd Level); C: EuKaryotic Orthologous Groups functional classification chart of differentially expressed proteins.

analysis between the expression levels of these three differentially expressed proteins and clinical indexes (Supplementary Table 1). We found that the serum alpha II B integrin protein levels were positively correlated with THCY (homocysteine) and FCP ($P < 0.05$, Figure 5B), while that of PCX was positively correlated with HOMA2-B and negatively correlated with HbAc1 and FBP ($P < 0.05$, Figure 5C). In addition, the serum protein levels of MCP were positively correlated with multiple parameters, including HbAc1, RBP, FCP, FINS, N-HDL, and HOMA2-IR, and negatively correlated with HOMA2-S% ($P < 0.05$, Figure 5D).

DISCUSSION

The SGLT-2 inhibitor dapagliflozin, as a new hypoglycemic drug, plays a role in lowering blood glucose by reducing the reabsorption of SGLT2 receptor glucose in renal tubular epithelial cells in patients with T2DM. Here, we performed label-free quantitative proteomics analysis of serum samples in patients before and after dapagliflozin treatments and identified differentially expressed proteins associated with dapagliflozin treatment. Notably, our function annotation and enrichment analysis suggested that three differential proteins (including αIIβ integrin, MPO, and PCX) potentially contribute to the renal and cardiovascular protective roles of dapagliflozin through participating in the regulation of multiple pathways. Furthermore, the serum differential expressions of these proteins were validated by ELISA, and their levels were correlated with some clinical indexes in patients with T2DM.

This study used dapagliflozin as the representative drug for SGLT-2 inhibitors. We found that the related indexes of islet function, such as FBG, HbA1c, FCP, FINS, and HOMA2-IR, decreased after dapagliflozin treatment. The lipid metabolism of T2DM patients was significantly improved after dapagliflozin treatment, as evidenced particularly by decreased non-HDL-C and increased ApoA1

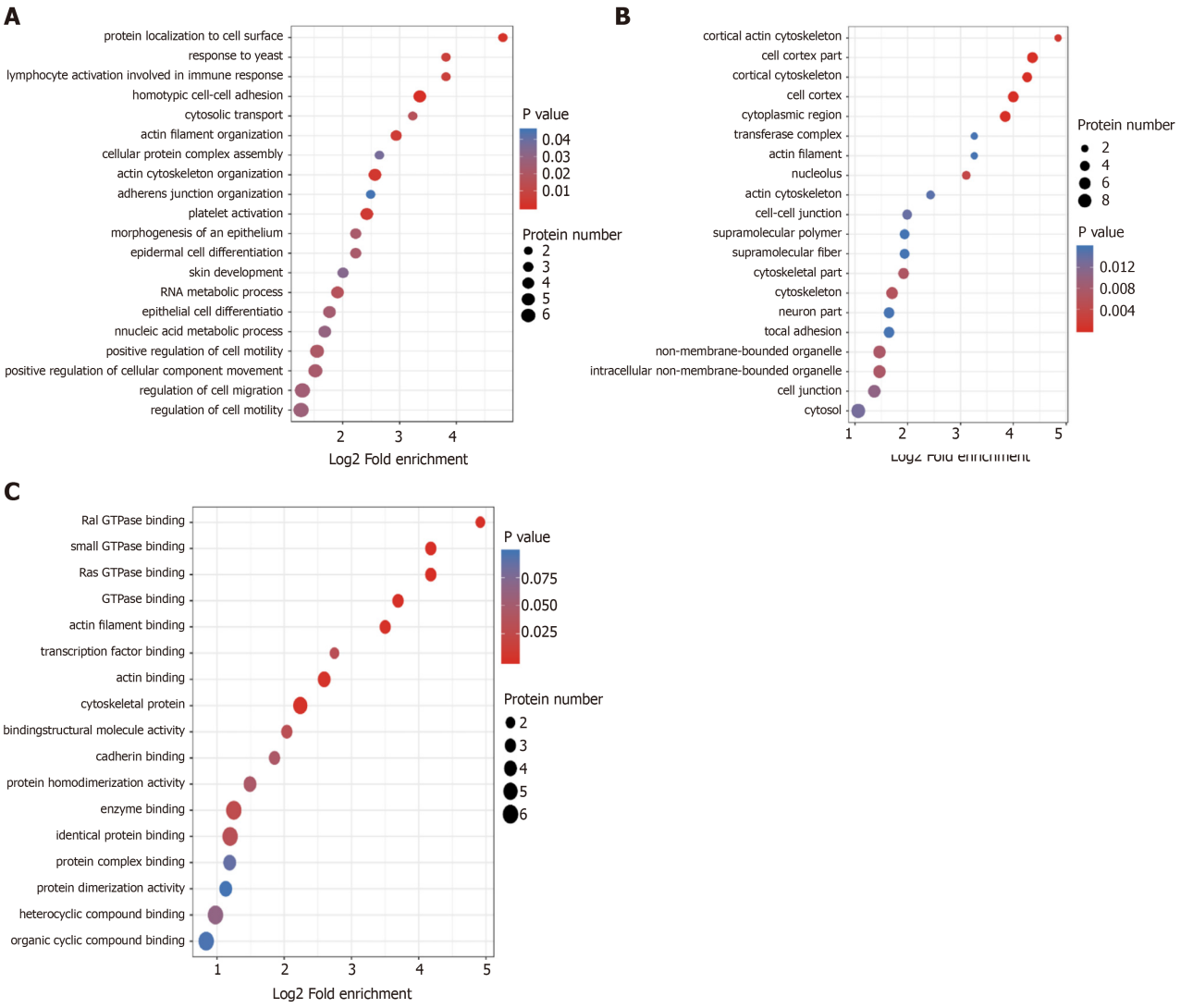

Figure 3 Gene Ontology enrichment bubble plots of differentially expressed proteins in three categories. A-C: The Gene Ontology enrichment bubble plots in the categories of biological process (A), cellular component (B), and molecular function (C) are shown. The bubble chart shows the results of the top 20 categories with the most significant enrichment. In the bubble chart, the vertical axis is the function classification or pathway, and the horizontal axis is the value after Log2 conversion of the ratio of the differential protein in the functional type compared to the ratio of the identified protein. The circle's color indicates the *P* value of enrichment significance, and the size of the circle indicates the number of differential proteins in the functional class or pathway.

Levels. Compared with baseline, the participants after dapagliflozin administration had significantly decreased BMI and hip circumference. We also found that RBP4, NEFA, and HCY were decreased after treatment with dapagliflozin. The controls were patients with mild T2DM in whom diet and exercise were sufficient to control their condition. Of note, dapagliflozin decreased FBG from 9.60 ± 2.44 to 6.80 ± 1.11 mmol/L, close to the value in controls (6.15 ± 0.67 mmol/L). Dapagliflozin also decreased FINS to lower levels than in controls, but HOMA2-B was lower than in controls, while there were no significant differences in HOMA2-S% and HOMA2-IR between the two groups. Compared with the controls, patients with dapagliflozin had higher apoA1 Levels and lower NEFA levels, also supporting the benefits of dapagliflozin. These results support the known effects of dapagliflozin in patients with T2DM[15]. RBP4, as a fat-derived factor, is closely related to obesity, insulin resistance, and other diseases[24]. Ost *et al*[25] found that RBP4 can prevent insulin-stimulated serine phosphorylation at position 307 of IRS1 by interfering with RBP4 and its antibodies in primitive adipocytes and correspondingly increasing the effective concentration of IRS1 tyrosine phosphorylation by half, as well as preventing the phosphorylation of ERK1/2. Therefore, it can be concluded that RBP4 might interfere with the Ras/MAPK signal of the insulin receptor by interfering with the phosphorylation of ERK1/2, thus participating in insulin resistance. In this study, after label-free quantitative proteomics, we also found that the differentially expressed proteins were significantly enriched in the Ras GTPase pathway. Since these two metabolic pathways share a common pathway, whether dapagliflozin could play a role through this pathway and the mechanism of RBP4 in dapagliflozin effectiveness need to be further studied.

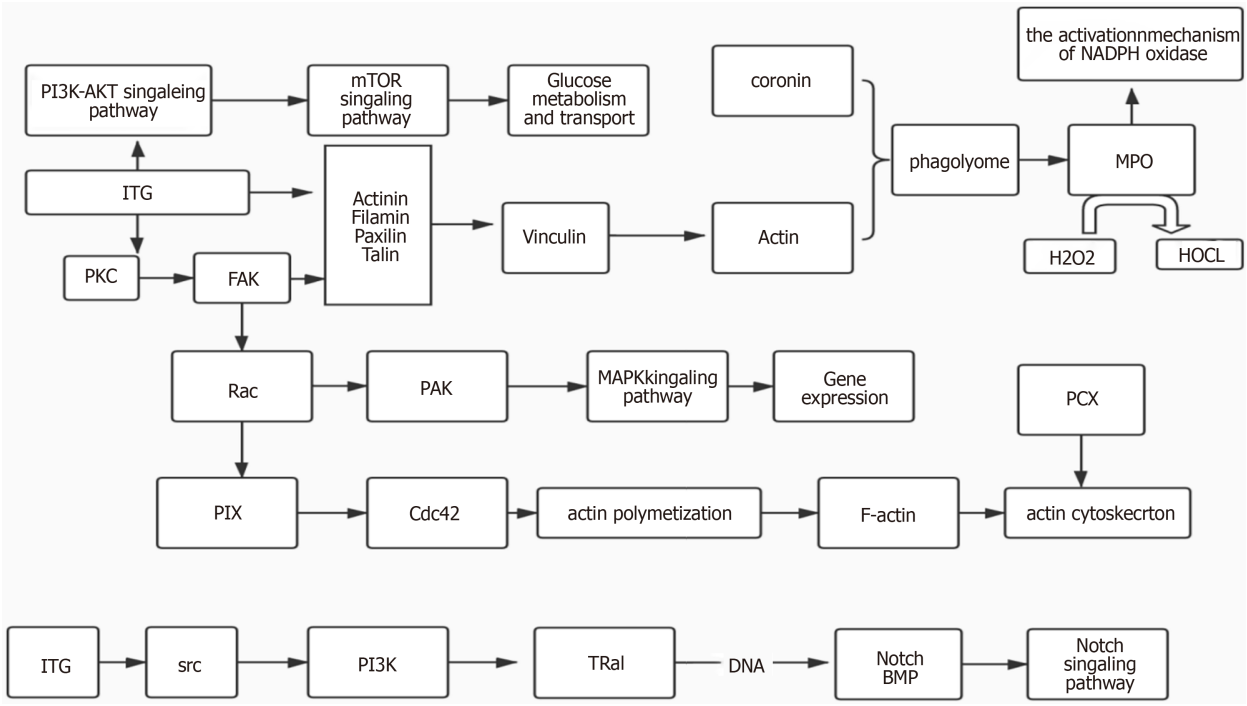

Figure 4 Treemap chart shows the interaction among differentially expressed proteins. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis identified a protein-protein interaction network among the integrin protein, myeloperoxidase, and podocalyxin.

Our comparative analysis with non-standard quantification demonstrated that 18 proteins were downregulated, and one was upregulated in serum samples of patients after dapagliflozin treatment. After consulting the literature, three candidate differential proteins closely related to T2DM were selected for ELISA validation. The differential expressions of these proteins were validated using serum samples from all the patients before and after dapagliflozin treatments. Notably, the correlation analysis suggested that the serum MPO protein levels were positively correlated with multiple clinical indexes, and the alpha II B protein levels were positively correlated with THCY and FCP levels. In addition, the serum PCX levels were negatively correlated with HOMA2-B and positively correlated with HbA1c and FBP. Based on these results and the literature, these three differential proteins might contribute to the beneficial roles of dapagliflozin in treating T2DM patients. Of note, PCX is the main surface antigen of podocytes and is normally expressed in renal podocytes, endothelial cells, and vascular endothelial cells and participates in maintaining the vascular endothelial cell barrier and reducing vascular inflammation. High glucose levels downregulate the expression of PCX in cultured podocytes *via* ERK1/2 MAPKs and inhibit the expression of PCX protein and mRNA by WT1 tumor protein and advanced glycation end-products[26], possibly resulting in the reduction of PCX in the blood. Second, we agree that integrins are cellular proteins that would not be expected to be found in circulation. Still, this study was not designed to determine the source of these proteins in the plasma. On the other hand, numerous studies report serum/plasma levels of various integrins as markers of diseases[27-29]. It could be hypothesized that the systemic inflammatory condition observed in T2DM increases cell death, releasing those proteins in circulation, but the present study cannot provide an answer regarding that point. Future studies will have to examine that specifically.

MPO is a heme enzyme and is the major protein in neutrophils and, to a lesser extent, in monocytes. MPO uses H_2O_2 to generate HOCl, a potent bactericidal agent, generating ROS[30]. MPO plays an essential part in the innate immune system by catalyzing the production of HOCl[31], but MPO has also been implicated as a very harmful agent in an increasing number of inflammatory-mediated disorders [32]. It has been reported that MPO is related to insulin resistance and inflammation parameters in overweight subjects with first-degree relatives of T2DM[31]. In addition, plasma MPO levels were positively correlated with the degree of coronary artery stenosis in T2DM patients, and increasing blood glucose might amplify the association between MPO and coronary artery disease[33]. Patients with uremic diabetic nephropathy with a low MPO level might be at a lower risk for any cardiac event than uremic patients with high MPO levels, suggesting that MPO might be a biomarker to predict coronary events in diabetic patients end-stage renal disease[34]. In this study, MPO levels in the dapagliflozin group decreased after treatment, related to the improvement of blood glucose control and inflammatory oxidation. Since our correlation analysis suggested that MPO was positively correlated with indexes including RBP, FCP, FINS, N-HDL, and HOMA2-IR, MPO very likely participated in oxidative stress regulation and acute, chronic inflammation of T2DM through a certain metabolic pathway. However, the specific mechanisms of MPO in contributing to the beneficial roles of dapagliflozin are still under

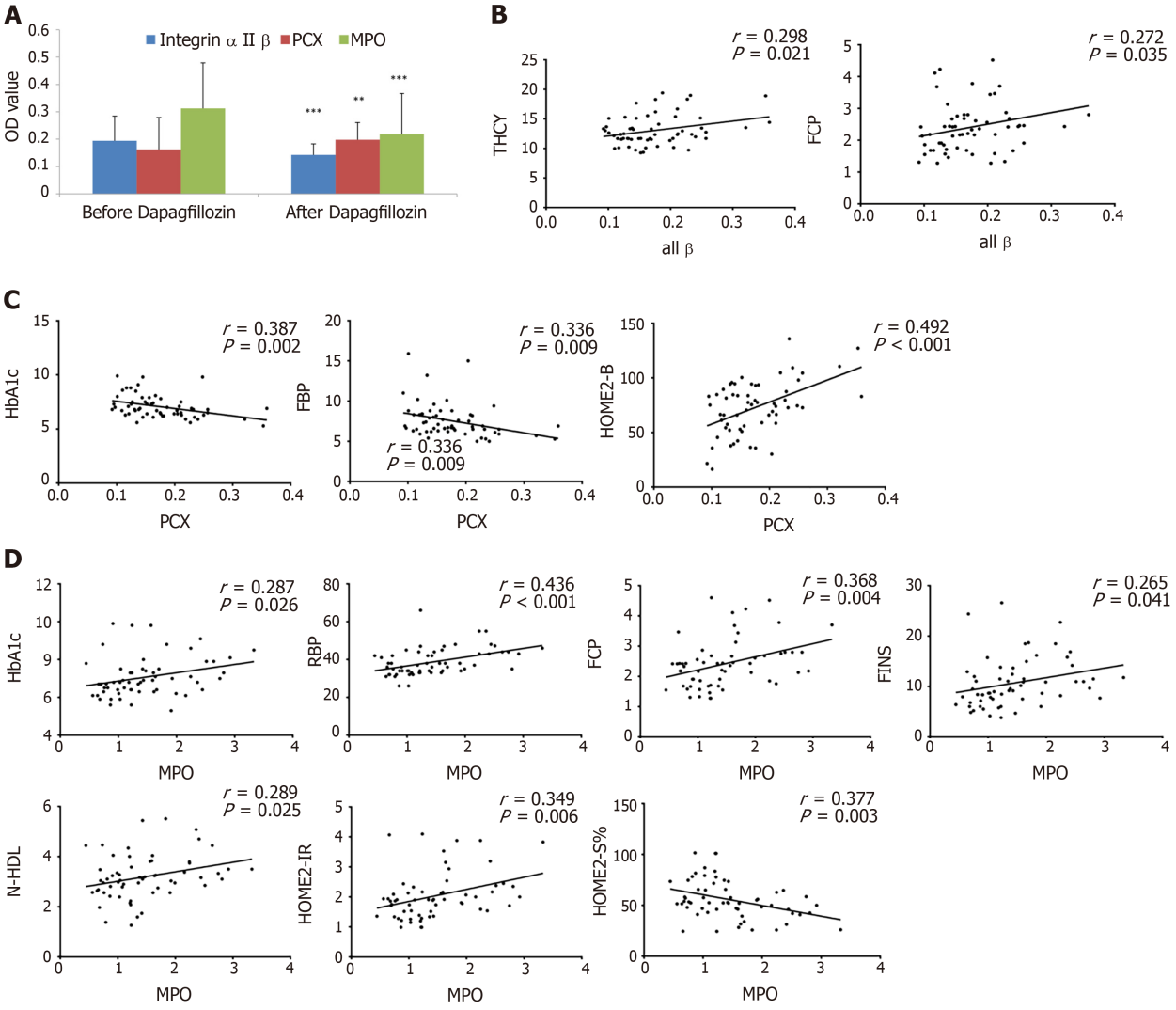

Figure 5 Correlation analyses of the expression levels of three differentially expressed proteins and clinical indexes of type 2 diabetic patients. A: The levels of three differentially abundant proteins, including myeloperoxidase (MPO), alpha II B integrin, and podocalyxin (PCX) proteins in serum samples of patients before and after dapagliflozin treatments were evaluated by enzyme linked immunosorbent assay. $n = 20$ for each group. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, between the indicated groups; B-D: Dot plots show the Pearson correlations between the levels of alpha II B integrin (B), PCX (C), and MPO (D) proteins and some clinical indexes in patients before and after dapagliflozin treatments.

investigation.

Integrin is a transmembrane protein that exists on the cell membrane to mediate cell-to-cell interaction. It can interact with various growth factors at the receptor level and regulate cell adhesion, survival, growth, differentiation, proliferation, and migration[35]. There are 24 kinds of integrins in humans, formed by heterodimerization of 18 alpha subunits and eight beta subunits. Alpha II beta integrin is one of the important components of the integrin family[35]. Studies have confirmed that platelet apoptosis in patients with T2DM is higher than in normal patients, increasing alpha II beta integrin levels[36]. The mechanism might be that integrins participate in RAS/MAPK signal transduction through interaction with growth factors and synergism between integrins, related to insulin resistance and secretion deficiency[37,38]. It has not been reported through which pathway dapagliflozin can induce the downregulation of alpha II beta integrin. Our analysis indicated that the Ras GTP pathway enrichment might be related to the downregulation of alpha II beta integrin in the differential protein function enrichment pathway in this study, which needs further confirmation by additional experiments.

The normal expression of PCX can prevent negatively charged proteins from leaking into human urine, resist adhesion between adjacent foot processes, and prevent adhesion between parietal epithelial cells and capillary loops[39]. Through animal experiments, Qi *et al*[40] demonstrated that high glucose could activate the ERK1/2 MAPK pathway of podocytes and decrease PCX expression. Although hyperglycemia inhibits PCX expression during glomerular injury, it can aggravate podocyte injury, increasing PCX with urine excretion[40]. These results suggest that urinary PCX is increased in urinary nephropathy, but PCX expression decreases with high glucose levels. We found that PCX was

negatively correlated with HbA1c and FBP. The lipid metabolism disorders in diabetic nephropathy were higher than in diabetic patients without nephropathy, and there were obvious disorders in the early stage of diabetic nephropathy (Figure 5B). This study suggests that dapagliflozin might participate in the protective effect of PCX protein in kidney through lipid metabolism-related mechanisms, and there are many pieces of evidence. Dapagliflozin has a protective effect on kidney by affecting glomerular feedback, restoring renal blood flow and glomerular filtration rate, thus preventing the progression of diabetic nephropathy in the initial stage, and posing potential renal protective effect on patients with mild to moderate renal insufficiency[13-15]. SGLT2 inhibitors can also limit the glycotoxicity of the kidney itself and reduce renal hypertrophy[13-15]. Vallon *et al*[41] suggested that SGLT2 inhibitors could inhibit the expression of inflammatory markers and fibrotic markers, and whether PCX was involved in these processes needs further study.

This study has several limitations. Because it was an exploratory study, no power analysis was initially performed, and the participants were enrolled using convenience sampling. Five serum samples with relatively small heterogeneity were directly selected from the dapagliflozin group for LC-MS. The abundance of proteins in the different samples was analyzed by comparing the frequency of mass spectrometry analysis or the peak intensity of mass spectrometry. Because a single sample can only be analyzed separately, the analytical flux was relatively low, and repeated experiments are needed to improve the accuracy of the analytical flux. Moreover, the sample size of this study is insufficient. Due to limited time and funding, the mechanisms of the differentially expressed proteins associated with dapagliflozin treatments need to be further studied. Many proteins' bioinformatics database data collection might be needed for analyzing the interaction between identified differentially expressed proteins and predicting the correlation between these differentially expressed proteins and clinical indicators. In addition, the relationship between dapagliflozin treatments and the changes of isoproteins still needs to be further validated.

CONCLUSION

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients. Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

ARTICLE HIGHLIGHTS

Research background

Only 50% of patients with type 2 diabetes mellitus (T2DM) can control their blood glucose levels. Dapagliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT-2) that improves the insulin sensitivity of the liver and peripheral tissues. Many studies confirmed that SGLT2 inhibitors reduce blood glucose and have multiple beneficial effects such as weight loss, lipid regulation, and kidney protection.

Research motivation

The mechanisms of the renal and cardiovascular protective effects of dapagliflozin from the perspective of differentially expressed proteins in the serum of T2DM patients have not been intensively explored so far.

Research objectives

This study aimed to identify differentially expressed proteins associated with dapagliflozin treatment in patients with T2DM. The results could help understand the mechanisms of dapagliflozin in patients with T2DM.

Research methods

Twenty T2DM patients [hemoglobin A1c (HbA1c) 7.0%-10.0%] were enrolled at The Affiliated Hospital of Inner Mongolia Medical University between January 1, 2017 and December 1, 2018. They received dapagliflozin (10 mg/d) for 3 mo, and the HbA1c < 7.0% target was achieved. The changes in clinical indexes were compared before and after treatments. Label-free quantitative proteomics was used to identify differentially expressed proteins using the serum samples of five patients. The identified differentially expressed proteins were analyzed using various bioinformatics tools.

Research results

Dapagliflozin significantly improved the clinical manifestation of the patients. There were 18 downregulated proteins and one upregulated protein in the serum samples of patients after dapagliflozin administration. Bioinformatics analyses, including subcellular localization, EuKaryotic Orthologous Groups, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes annotations, were used to profile the biological characteristics of the 19 differentially expressed proteins. Based on the literature and function enrichment analysis, two downregulated proteins, myeloperoxidase (MPO) and alpha II B integrin, and one upregulated protein, podocalyxin (PCX), were selected for enzyme linked immunosorbent assay (ELISA) validation. These validated differentially expressed proteins had multiple correlations with clinical indexes, including HbAc1 and fasting C-peptide.

Research conclusions

Dapagliflozin has obvious hypoglycemic effects, and it can also improve weight loss, lipid metabolism, and islet function of patients with T2DM. After dapagliflozin treatment, 18 proteins (including MPO and alpha II beta integrin) were downregulated, and PCX protein was upregulated in the serum of T2DM patients.

Research perspectives

Subsequent function annotation and enrichment analysis, as well as ELISA validation and correlation analysis with the clinical indexes, suggested that MPO, alpha II beta integrin, and PCX might contribute to the beneficial roles of dapagliflozin through their regulations on oxidative stress, insulin resistance, and lipid metabolism.

FOOTNOTES

Author contributions: Zhao YX contributed to methodology, software, formal analysis, investigation, resources, data curation, writing original draft preparation, writing review and editing; Borjigin S contributed to experimental operation. Yan ZL contributed to conceptualization, methodology, validation, formal analysis, investigation, resources, writing original draft preparation, writing review and editing, supervision, funding acquisition; all authors have read and agreed to the published version of the manuscript.

Supported by Major Scientific Research Program of The Affiliated Hospital of Inner Mongolia Medical University, No. NYFY ZD 001.

Institutional review board statement: The present study was approved by the Ethics Committee of Affiliated Hospital of Inner Mongolia Medical University, No. WZ.

Informed consent statement: Written informed consent was obtained from the patients.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Wu RR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 240-250

DOI: 10.4239/wjd.v13.i3.240

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Higher risk of type 2 diabetes in young women with polycystic ovary syndrome: A 10-year retrospective cohort study

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Nong X, Popova PV

Received: August 12, 2021
Peer-review started: August 12, 2021
First decision: October 3, 2021
Revised: October 13, 2021
Accepted: February 19, 2022
Article in press: February 19, 2022
Published online: March 15, 2022

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Abstract

BACKGROUND

Polycystic ovary syndrome (PCOS) is a common disorder in women of reproductive age. Over the last few decades, research studies have revealed that PCOS is strongly associated with metabolic disorders, including metabolic syndrome, obesity, insulin resistance and prediabetes. Clinical observation has shown that women with PCOS are expected to have an increased risk of developing type 2 diabetes (T2DM) in the future.

AIM

To assess the hazard ratio (HR) of T2DM between women with/without PCOS.

METHODS

This population-based, retrospective cohort study evaluated data retrieved from the National Health Insurance Research Database. The subjects were women with PCOS (n = 2545) identified on the basis of diagnosis, testing, or treatment codes, and women without PCOS as controls (n = 2545). The HR of T2DM between women with or without PCOS was the main outcome measure analyzed.

RESULTS

Our study found that, during a 10-year follow-up period, the overall incidence of T2DM was 6.25 per 1000 person-years in the PCOS group compared with 1.49 in the control group. After adjustment for potential confounding variables, the overall incidence of T2DM was higher in the PCOS group *vs* the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18–24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. However, no such significant association was noted in women older than 35 years.

CONCLUSION
These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes in women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

Key Words: Polycystic ovary syndrome; Diabetes; Incidence; Hazard ratio; Population-based cohort study

Core Tip: We aimed to evaluate the incidence of type 2 diabetes (T2DM) over time in women with polycystic ovary syndrome (PCOS) at different diagnosis ages, in comparison with non-PCOS controls. Our results showed that, among women diagnosed with PCOS at a young age, the incidence of T2DM was significantly higher than that of age-matched women in the general population. However, the risk disappeared among women diagnosed with PCOS after age 35. These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

Citation: Liao WT, Huang JY, Lee MT, Yang YC, Wu CC. Higher risk of type 2 diabetes in young women with polycystic ovary syndrome: A 10-year retrospective cohort study. *World J Diabetes* 2022; 13(3): 240-250
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/240.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.240

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine pathology that affects 5%-15% of women of reproductive age worldwide. The prevalence estimates vary according to the different diagnostic parameters applied[1]. It is also the most common cause of endocrine-related female infertility in the United States. This syndrome was first described by American gynecologists Irving F, Stein Sr. and Michael L Leventhal in 1935, when they reported a series of patients with enlarged polycystic ovaries, hirsutism, oligo/amenorrhea, and infertility. It has been demonstrated that PCOS includes a complex of systemic symptoms in addition to the reproductive apparatus. Over the last few decades, research studies have revealed that PCOS is strongly associated with metabolic disorders, including metabolic syndrome, obesity, insulin resistance, prediabetes, and type 2 diabetes (T2DM). The prevalence of metabolic syndrome in women with PCOS was approximately 6- to 7-fold higher than that detected in the controls[2,3]. According to a prospective case–control study, 64.4% of 271 patients with PCOS were noted to be insulin-resistant after adjusted for age, race, and body mass index (BMI)[4]. Based on clamp data, both obese and lean women with PCOS were more insulin-resistant compared with their weight-matched normal counterparts. In this study, insulin resistance (IR) was present in 75% of lean women with PCOS, 62% of overweight controls, and 95% of overweight women with PCOS[5]. Insulin resistance is defined as a reduced response of target tissues, such as the skeletal muscle and adipocytes. In women with PCOS, insulin-mediated glucose uptake is decreased by 35%-40% compared with age- and weight-comparable control women[6]. Because insulin resistance is the driving factor of hyperglycemia, women with POCS are particularly at risk of developing T2DM. The estimated prevalence of impaired glucose tolerance (IGT) and T2DM was 31%-37% and 7.5%-10.0%, respectively, in women with PCOS in the United States[7-9]. In two prospective trials of women with PCOS conducted in the United States and Turkey, after an average follow-up period of 2-3 years, both studies revealed a higher IGT and T2DM conversion rate compared with women without PCOS[9,10]. Abundant strong evidence supports the contention that diabetes is much more prevalent in women with PCOS than it is in the general population. We noticed that, even at a young age, women with PCOS also exhibit β-cell dysfunction, IGT, and T2DM[11,12]. Therefore, we aimed to evaluate the incidence of T2DM over time in women with PCOS at different diagnosis ages, in comparison with non-PCOS

controls. We selected the National Health Insurance Research Database (NHIRD), which records age, gender, diagnosis codes, comorbidities, and the clinical prescriptions for each beneficiary, as the data source.

MATERIALS AND METHODS

Data source

In this population-based retrospective cohort study, we used data from individuals in the Longitudinal Health Insurance Database 2000 (LHID2000), to evaluate the outcomes. LHID2000 is a subset of the NHIRD that contains the entire original claim data of 1000000 individuals randomly sampled from the 2000 registry for beneficiaries (ID) of the NHIRD, which maintains the registration data of everyone who was a beneficiary of the National Health Insurance (NHI) program during the period of 1996-2013. There are approximately 23.75 million individuals in this registry. The complete registration and claim data of these 1000000 individuals collected by the NHI program constitute the LHID2000. There was no significant difference in the gender distribution (χ^2 = 1.74, df = 1, *P* value = 0.187) between the patients in the LHID2000 and those in the original NHIRD[13]. The data recorded in the LHID2000 include demographic information; prescription details; clinical events; diagnosis codes in accordance with the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) system; and medical examinations and managements for all admitted patients and outpatients. In this study, we used LHID2000 from 1997 to 2010 as the research database, and followed to December 2013. This study was approved by the institutional review board of China Medical University in central Taiwan (CMUH104-REC2-115).

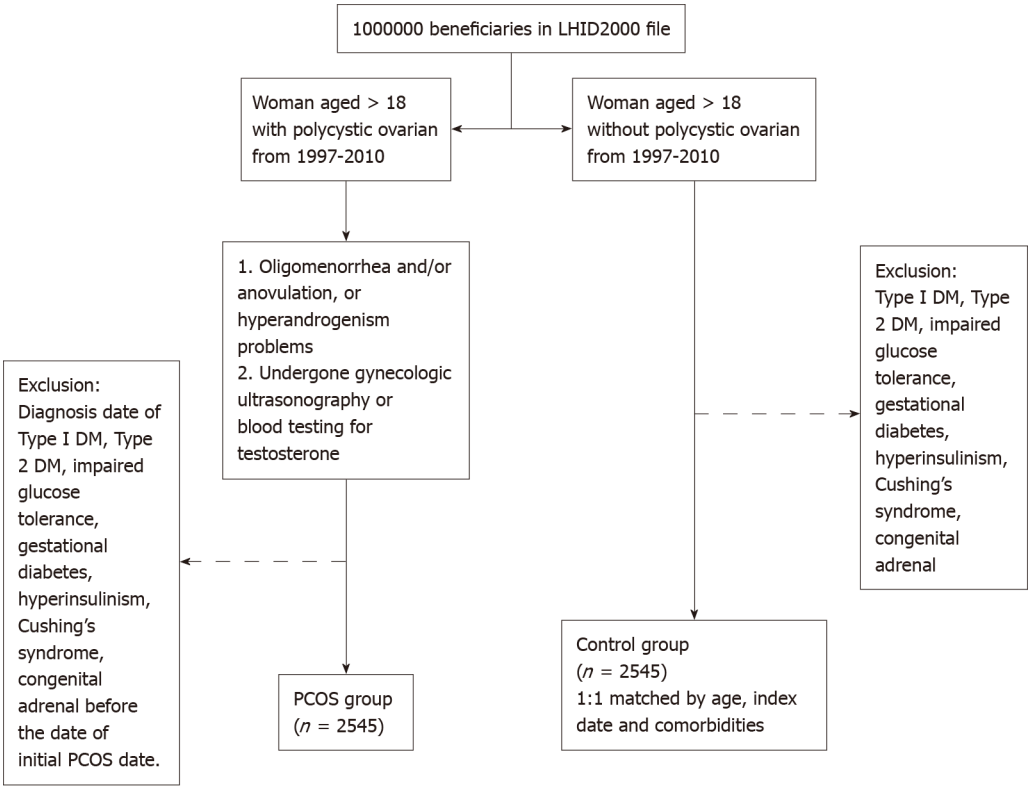
Study population and outcome assessment

In this retrospective cohort study, we aimed to compare women with PCOS with those without PCOS. We selected the PCOS cohort as follows: (1) Women older than 18 years of age and diagnosed with polycystic ovarian morphology; (2) Clinical visit for oligomenorrhea and/or anovulation problems, or hyperandrogenism problems at least twice a year; and (3) Women who underwent gynecological ultrasonography or blood testing for testosterone or 17-hydroxyprogesterone levels. Women were eligible to participate when all three conditions were met. Patients with the diagnoses of type 1 diabetes, T2DM, IGT, gestational diabetes, hyperinsulinism, Cushing's syndrome, and congenital adrenal hyperplasia before the date of initial PCOS diagnosis and those who were aged less than 18 years were excluded from the cohort. According to the inclusion and exclusion criteria, a total of 2545 people were defined as the PCOS group in this study (Figure 1). The factors associated with PCOS that are considered as potential confounding variables include lipid metabolism disorders, coronary artery disease, hypertension, chronic kidney disease, cerebrovascular accident, female infertility, obesity, chronic lymphocytic thyroiditis, major depression, and a history of anxiety before baseline. Prescriptions during follow-up for menstrual cycle regulation, ovulation induction, anti-androgen, and metformin were also considered potential confounding variables. The index date for the cohort group was assigned as the first time of recording of the ICD-9-CM code. The end point was set on the date of the new diagnosis of T2DM (more than three times at outpatient department or once in admission), the date of withdrawal from the NHI program, or the end of 2013. For the control group, women without PCOS were randomly selected and 1:1 frequency matched the cohort group by age, index date, and comorbidities. The comorbidities controlled in this study were lipid metabolism disorders, hypertension, coronary artery disease, chronic kidney disease, cerebrovascular accident, infertility, obesity, Hashimoto's disease, major depression, and anxiety.

Statistical analysis

The baseline characteristics of women with PCOS and controls are described by numbers and percentages. An intergroup comparison was performed using the chi-squared and *t*-test for categorical variables and continuous variables, respectively. The incidence rates of T2DM were calculated in person-years. We used univariable and multivariable Cox proportional hazard regression models to estimate and adjust the crude hazard ratio (HR). After adjustment for key covariates (age, comorbidity), we calculated the adjusted HR together with 95%CIs with statistical significance set at $P < 0.05$. Survival curves were estimated for each group, considered separately using the Kaplan–Meier method and compared statistically using the log-rank test. The Kaplan–Meier curves of the cumulative incidence of T2DM between the PCOS group and the control group were performed to estimate the cumulative probability of T2DM between two groups. All analyses were performed using the SAS software (version 9.4 for windows; SAS Institute, Cary, NC, United States).

The study was reviewed by our expert biostatistician Dr. Jing-Yang Huang.



DOI: 10.4239/wjd.v13.i3.240

Figure 1 Flow diagram of the recruitment of subjects from the 1 million samples randomly selected from the Taiwanese National Health Insurance Research Database from 1997 to 2010. LHID2000: Longitudinal Health Insurance Database 2000; PCOS: Polycystic ovary syndrome.

RESULTS

The first possible date for cohort entry (the study start date) was January 1, 1997, and patients could enter the cohort until December 31, 2010. The end of the follow-up time is December 2013. During the study period, we identified 2545 women with PCOS. These women were frequency matched at 1:1 to 2545 individuals in the non-POCS control group. The PCOS group and the control group were both followed for a mean period of 10 years, and the standard deviation was 3.14 *vs* 3.15 years (Table 1). In the baseline characteristic of the patients, all enrollees are stratified by age. The highest proportion of patients were into the 18-24-year age group (58.8%), followed by the 25-29-year age group (22.9%). The proportion of women over 30 years of age was only 18.3%. As expected, the age and comorbidity distributions of these two groups were similar because the groups were 1:1 propensity-score matched. Their mean age was 25 years, and there was no difference in the age stratification between the two groups. Women with PCOS were more likely to receive a prescription of metformin, oral contraceptive pills (OCPs), clomiphene citrate, and spironolactone.

In the PCOS group, the overall incidence of T2DM was 6.25 per 1000 person-years compared with 1.49 in the control group (Table 2). After adjustment for potential confounding variables (age, all comorbidities, and the medications listed in Table 1), the incidence of T2DM was higher in the PCOS group compared with the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). Moreover, the PCOS group showed a higher incidence of T2DM in the 18-24-year age group (HR = 10.4, 95%CI: 5.04-21.4, $P < 0.0001$). The incidence of T2DM decreased with the increasing diagnosis age. However, no such significant association was noted in women older than 35 years. All participants were stratified according to the presence or absence of comorbidities or of medication. Among women without comorbidities and no medication, the PCOS group exhibited a higher incidence of T2DM compared with the control group (non-comorbidity stratifications: adjusted HR = 7.62, 95%CI: 4.68-12.4; non-metformin stratifications: adjusted HR = 5.41, 95%CI: 3.67-7.98; non-OCP stratifications: adjusted HR = 5.18, 95%CI: 3.54-7.58; non-clomiphene stratifications: adjusted HR = 5.93, 95%CI: 3.94-8.92; non-spironolactone stratifications: adjusted HR = 5.07, 95%CI: 3.47-7.41). The Kaplan–Meier curves present the differences in the cumulative incidence of T2DM between the PCOS group and the control group (Figure 2). The cumulative incidence of T2DM in the PCOS group (dashed line) was significantly higher than that observed in the control group (solid line) (log-rank test, $P < 0.001$).

Table 1 Baseline patient characteristics

	PCOS				
	Yes (*n* = 2545)		No (*n* = 2545)		*P* value
	n	%	*n*	%	
Age, yr					> 0.99
18-24	1497	58.8	1497	58.8	
25-29	583	22.9	583	22.9	
30-34	289	11.4	289	11.4	
35-39	117	4.60	117	4.60	
40-44	45	1.77	45	1.77	
≥ 45	14	0.55	14	0.55	
mean ± SD	25.1 ± 5.81		25.2 ± 5.91		0.63
Comorbidity					
Disorders of lipid metabolism	38	1.49	38	1.49	> 0.99
Cardiovascular disease	3	0.12	8	0.31	0.13
Hypertension	27	1.06	27	1.06	> 0.99
Chronic kidney disease	2	0.08	4	0.16	0.41
Cerebrovascular accident	7	0.28	8	0.31	0.80
Infertility	151	5.93	151	5.93	> 0.99
Obesity	27	1.06	27	1.06	> 0.99
Hashimoto's disease	5	0.20	4	0.16	0.74
Major depression	18	0.71	27	1.06	0.18
Anxiety	169	6.64	169	6.64	> 0.99
Medication (during follow-up period)					
Metformin	238	9.35	18	0.71	< 0.0001
OCPs	443	17.4	72	2.83	< 0.0001
Clomiphene	1384	54.4	302	11.9	< 0.0001
Spironolactone	111	4.36	32	1.26	< 0.0001

Polycystic ovary syndrome group: follow-up time: 10.0; SD = 3.14. Control group: follow-up time: 10.0; SD = 3.16. PCOS: Polycystic ovary syndrome; OCPs: Oral contraceptive pills.

DISCUSSION

To our knowledge, this was the first attempt to analyze large-scale data to evaluate the relationship between women with PCOS and the development of T2DM in an East-Asian cohort. Moreover, this was the only study that stratified the cohorts into subgroups based on the age at diagnosis.

Our study found that, during a 10-year follow-up period, women with PCOS were associated with 5-fold higher risk of developing T2DM compared with women without PCOS. In past studies, the incidence of T2DM in women with PCOS presented with substantial clinical heterogeneity (ranging from 2- to 7-fold). There may be several explanations for these marked differences. First, different ethnic backgrounds may be responsible for the higher prevalence of T2DM. A small-size prospective trial carried out in the eastern Mediterranean region showed that 11.5% of women with PCOS and normal glucose tolerance (NGT) at the baseline converted to IGT with an annualized incidence rate of 4.5%. Furthermore, the annualized incidence rate from IGT converted to T2DM was 10.4%. In comparison, another similar study conducted in the United States reported that, among women with PCOS, the annualized conversion risk was 16% from NGT to IGT, and 2% from IGT to T2DM[9,10]. A nationwide population-based retrospective cohort study performed in Denmark found that the HR for women with PCOS who developed T2DM was 3.5 (95%CI: 3.2-3.8) when gestational diabetes mellitus was excluded. The results of the Danish study were slightly lower than our current findings (HR = 5.13, 95%CI: 3.51-

Table 2 Incidence rate and hazard ratio of type 2 diabetes between two groups stratified by gender, age, and comorbidity

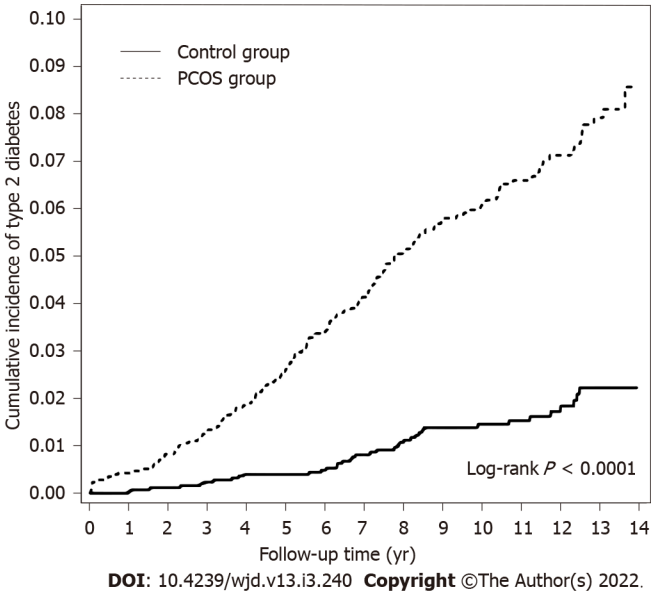
	PCOS									
	No			Yes			Crude		Adjusted	
	Event	PY	IR	Event	PY	IR	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Overall	38	25483	1.49	159	25460	6.25	4.19 (2.94, 5.97)	< 0.0001	5.13 (3.51, 7.48)	< 0.0001
Age										
18-24	9	15453	0.58	74	15301	4.84	8.33 (4.17, 16.6)	< 0.0001	10.4 (5.04, 21.4)	< 0.0001
25-29	9	5630	1.60	40	5766	6.94	4.32 (2.10, 8.90)	< 0.0001	5.28 (2.42, 11.5)	< 0.0001
30-34	9	2753	3.27	27	2734	9.88	3.01 (1.42, 6.40)	0.004	4.06 (1.73, 9.53)	0.001
35-39	6	1117	5.37	11	1134	9.70	1.81 (0.67, 4.90)	0.24	2.14 (0.72, 6.35)	0.17
40-44	5	399	12.53	5	397	12.59	1.03 (0.30, 3.55)	0.97	1.68 (0.38, 7.41)	0.50
≥ 45	0	132	0.00	2	128	15.63				
Comorbidity[1]										
Yes	17	3864	4.40	32	3835	8.34	1.90 (1.06, 3.43)	0.03	2.14 (1.14, 3.99)	0.02
No	21	21620	0.97	127	21625	5.87	6.05 (3.81, 9.60)	< 0.0001	7.62 (4.68, 12.4)	< 0.0001
Medication										
Metformin										
Yes	2	191	10.47	21	2325	9.03	0.88 (0.21, 3.75)	0.86	0.54 (0.1, 2.78)	0.46
No	36	25292	1.42	138	23135	5.96	4.19 (2.91, 6.05)	< 0.0001	5.41 (3.67, 7.98)	< 0.0001
OCPs										
Yes	0	789	0.00	17	4559	3.73				
No	38	24695	1.54	142	20901	6.79	4.42 (3.09, 6.32)	< 0.0001	5.18 (3.54, 7.58)	< 0.0001
Clomiphene										
Yes	5	3349	1.49	72	14470	4.98	3.41 (1.38, 8.43)	0.008	3.26 (1.3, 8.21)	0.01
No	33	22135	1.49	87	10990	7.92	5.33 (3.57, 7.95)	< 0.0001	5.93 (3.94, 8.92)	< 0.0001
Spironolactone										
Yes	0	349	0.00	4	1211	3.30				
No	38	25135	1.51	155	24249	6.39	4.23 (2.97, 6.04)	< 0.0001	5.07 (3.47, 7.41)	< 0.0001

[1]Patients with any one of comorbidity were classified as the comorbidity group.

Models adjusted by age, all comorbidities and medications listed in Table 1. PY: Person-years; IR: Incidence rate, per 1000 person-years; HR: Hazard ratio; PCOS: Polycystic ovary syndrome; OCPs: Oral contraceptive pills.

7.48)[13]. The different ethnic backgrounds may be responsible for the higher prevalence of T2DM detected in Taiwan. A meta-analysis of multiple quality studies calculated an increased prevalence of IGT and T2DM among women with PCOS and different ethnicities (OR for IGT, Asia = 5.22, Americas = 4.4, Europe = 2.59)[14]. Genome-wide association studies (GWASs) have become a feasible option for studying the genetic background of PCOS, thus providing the ability of surveying a large number of genomes at once[15]. Two GWASs targeting PCOS have been performed in China; they identified 11 variants associated with PCOS risk in Han Chinese women who were diagnosed with PCOS (*i.e.*, who fulfilled all three Rotterdam criteria)[16,17]. However, not all loci for PCOS have been replicated in European women, which may speak to the variation in susceptible single-nucleotide polymorphisms (SNPs) among distinct racial and ethnic groups[18]. Some researchers believe that different combinations of SNPs may underlie the severity of the PCOS phenotypes, with Americans and Asians being more often characterized by the metabolic phenotype, and Europeans and Middle-Eastern women having a higher prevalence of hyperandrogenic phenotype[19]. Therefore, we assume that ethnicity may affect the transition from PCOS to diabetes[14].

Second, it may be related to the age at diagnosis of PCOS. This was also the most important finding of our study. There are indications that age may affect the incidence rate of conversion from PCOS to T2DM. According to a prospective study with a follow-up of 18 years performed in the United States, 53



DOI: 10.4239/wjd.v13.i3.240

Figure 2 Kaplan–Meier curves for the cumulative incidence of type 2 diabetes of the polycystic ovary syndrome and control groups.
PCOS: Polycystic ovary syndrome.

women fulfilled the criteria for PCOS at ages 20-32 (average, 26 years). Compared with those without PCOS, women with persistent PCOS had a 7-fold odds of developing diabetes[20]. Another 10-year follow-up study performed in China among women with PCOS aged 30-39 years reported that the age-standardized incidence rate of T2DM was approximately 3-fold compared with women without PCOS [21]. It is well established that the PCOS phenotype changes with aging, the improvement of phenotype, and oligo-ovulation, as indicated by the decrease in serum androgen levels (*e.g.*, testosterone, free androgen index, calculated free testosterone, androstenedione, and dehydroepiandrosterone sulfate) and increase in the number of regular menstrual cycles[22-25]. In healthy women, the positive correlation between age and worsening glucose tolerance is obvious after adjusting for BMI[26]. Interestingly, not only ovarian dysfunction and hyperandrogenism, but also insulin resistance, ameliorate during aging in women with PCOS[20]. According to a cross-sectional study, homeostasis model assessment (HOMA)-IR was negatively associated with age in women with PCOS as well as in different BMI subgroups, namely lean, normal-weight, and overweight subjects[27]. The observations that BMI and androgens are positively associated with HOMA-IR and that androgens decline with time suggest that these women achieved a better metabolic profile at their late reproductive ages. In a long-term prospective cohort study with a follow-up of more than 10 years, Kazemi Jaliseh *et al*[28] found that the adjusted HR for T2DM in women with PCOS aged ≤ 40 years was 4.9. In contrast, there was no difference between the two groups regarding the incidence rates of T2DM after the age of 40 years. The study included 178 women with PCOS and 1524 women without PCOS, and all PCOS cases were defined using the National Institutes of Health 1990 criteria, which carry the strongest clinical significance. The hazard differences between women with PCOS and those in the general population disappeared in their late reproductive years, which is in line with the results of the current study. Women who were diagnosed with PCOS before the age of 25 were 10 times more likely to develop T2DM compared with women without PCOS after adjusting for variance. The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18-24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. Although the risk decreased with increasing age, it remained higher compared with that detected in women without PCOS. After age 35, the association between PCOS and T2DM was not statistically significant. Furthermore, among women without comorbidities and taking no medications, the incidence of T2DM was higher in the PCOS group than that in the control group. Several reasons for this result have been identified. First, women with PCOS who had no comorbidities showed a higher incidence of T2DM than the overall average, which means that the health problems caused by PCOS may be higher than previously recognized. Second, women without comorbidities and taking no medications may be relatively younger, which corroborates the previous assumption that women who are diagnosed with PCOS at a young age are more likely to develop T2DM. However, the sample size in the stratification of no medication is notably very small and may not provide reliable estimates and conclusive results.

The strength of our study consisted in the fact that NHIRD is one of the largest and most comprehensive nationwide population reimbursement databases in the world, as it covers almost 23 million residents in Taiwan with universal coverage. It provides a big sample size and complete records of medical visits and treatment, which are conducive to a longitudinal study design and age stratification.

Furthermore, research conducted using NHIRD can avoid a selection bias and the possibility of recall bias in questionnaire assessments.

The limitation of this study was that certain prognostic factors that are associated with the incidence of T2DM are not available through the NHIRD; namely, BMI, waist-hip ratio, lifestyle, and the results of blood tests (androgen and plasma glucose levels). Thus, we were unable to rule out the possibility that the differences in HR detected between the two groups stemmed from these factors. Moreover, NHI covers 96%-99% of Taiwan's population and 93% of hospitals and clinics are NHI-contracted. It subsidizes most medical treatments at a relatively low cost. However, there is still a possibility that patients reviewed in this study might have consulted other doctors before entering the NHI system. In addition, the sample size of the groups of women diagnosed with PCOS after the age of 35 years was relatively small, which may have led to imprecise estimates and statistical significance. Finally, the study population was homogeneous because all women were Asian. Therefore, additional research is required to substantiate this association among non-Asian women as well.

CONCLUSION

The data supplied here were from a relatively large population, spanning a long period. Our results showed that, among women diagnosed with PCOS at a young age, the incidence of T2DM was significantly higher than that of age-matched women in the general population. However, the risk disappeared among women diagnosed with PCOS after age 35. These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

ARTICLE HIGHLIGHTS

Research background

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy in women of reproductive age. Research over the last few decades has revealed that PCOS is strongly associated with metabolic disorders. Even at a young age, women with PCOS also exhibit β-cell dysfunction, impaired glucose tolerance, and type 2 diabetes (T2DM).

Research motivation

Although current evidence supports the contention that diabetes is much more prevalent in women with PCOS than it is in the general population. The majority of longitudinal studies regarding the incidence of T2DM in women with PCOS are from non-Asian countries.

Research objectives

We aimed to evaluate the incidence of T2DM over time in women with PCOS at different diagnosis ages, in comparison with non-PCOS controls.

Research methods

The data retrieved from the Longitudinal Health Insurance Database 2000 (LHID2000). LHID2000 is a subset of the National Health Insurance Research Database (NHIRD) that contains the entire original claim data of 1000000 individuals randomly sampled from the 2000 registry for beneficiaries (ID) of the NHIRD, which maintains the registration data of everyone who was a beneficiary of the National Health Insurance program.

Research results

After adjustment for potential confounding variables (age, comorbidities and medications), the overall incidence of T2DM was higher in the PCOS group compared with the control group (HR = 5.13, 95%CI: 3.51-7.48, $P < 0.0001$). The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age: the adjusted HR was 10.4 in the 18-24-year age group, 5.28 in the 25-29-year age group, and 4.06 in the 29-34-year age group. After age 35, the association between PCOS and T2DM was not statistically significant.

Research conclusions

The risk of developing T2DM subsequent to PCOS decreased with increasing diagnosis age. No such significant association was noted in women older than 35 years.

Research perspectives

These findings highlight the importance of prompting a more aggressive treatment to prevent diabetes among women diagnosed with PCOS at a young age, and, in contrast, the lessened importance of this type of intervention in women diagnosed with PCOS at a late reproductive age.

ACKNOWLEDGEMENTS

This study was based in part on data from the National Health Insurance Research Database, provided by the National Health Insurance Administration, Ministry of Health and Welfare and managed by National Health Research Institutes. The interpretation and conclusions contained herein do not represent those of the National Health Insurance Administration, Ministry of Health and Welfare, or National Health Research Institutes.

FOOTNOTES

Author contributions: Liao WT and Wu CC contributed to the conceptualization, writing-review and editing; Huang JY, Lee MT and Yang YC contributed to the methodology; Yang YC contributed to the software and formal analysis; Liao WT and Lee MT contributed to the validation; Huang JY contributed to the data curation; Liao WT contributed to the writing-original draft preparation; Wu CC contributed to the supervision and project administration; all authors reviewed the final version of the manuscript.

Supported by the Show Chwan Memorial Hospital, No. SRD-108014.

Institutional review board statement: This study was approved by the institutional review board of China Medical University in central Taiwan (CMUH104-REC2-115).

Informed consent statement: The informed consent form is waived by the Institutional Review Board.

Conflict-of-interest statement: The authors declare no conflict-of-interest.

Data sharing statement: Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality and because they were used under license. Upon request, the corresponding author will provide details on the restrictions and conditions under which access to some data may be provided.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 251-259

DOI: 10.4239/wjd.v13.i3.251

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Hemoglobin within normal range is negatively related to hemoglobin A1c in a nondiabetic American population aged 16 years and older

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Specialty type: Biochemical research methods

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Darenskaya MA, Scaramuzza A

Received: September 19, 2021
Peer-review started: September 19, 2021
First decision: November 8, 2021
Revised: December 6, 2021
Accepted: February 20, 2022
Article in press: February 20, 2022
Published online: March 15, 2022

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Abstract

BACKGROUND

Protein glycosylated hemoglobin, hemoglobin A1c (HbA1c) binds hemoglobin (Hb) in red blood cells to blood glucose. However, the relationship between Hb and HbA1c remains unclear.

AIM

To elucidate their relationship in a nondiabetic population aged ≥ 16 years in the United States, using data from the 1999-2018 National Health and Nutrition Examination Survey.

METHODS

This study was based on data from 44560 adults aged ≥ 16 years, excluding those with diabetes. The relationship was estimated using a multivariate regression. We also used piecewise linear regression for subgroup analysis based on age and sex stratification and analysis of the threshold effects of Hb on HbA1c.

RESULTS

Hb and HbA1c levels were negatively correlated in the unadjusted model (β = -0.01; 95%CI: -0.01, -0.01). The correlation was significantly negative when the regression model was minimally regulated and stratified by age and sex, and remained negative when the model was further regulated (more than 10%) to identify covariates with the HbA1c level influence estimates. In subgroup analyses based on age and sex stratification, the association remained negative when the covariates were controlled. A nonlinear relationship was observed between them when the Hb levels reached the tipping point (13.2 g/dL) (adjusted odds ratio, -0.04; 95%CI: -0.05, -0.03) and when the Hb levels exceeded 13.2 g/dL (adjusted odds ratio, -0.10; 95%CI: -0.10, -0.09).

CONCLUSION
Our study shows that normal Hb levels are negatively correlated with HbA1c in nondiabetic Americans aged ≥ 16 years.

Key Words: Haemoglobin; Glycosylated haemoglobin; Diabetes; National Health and Nutrition Examination Survey

Core Tip: Our research revealed that hemoglobin (Hb) within the normal values is negatively related to hemoglobin A1c (HbA1c) in non-diabetic American populations aged 16 years and older. HbA1c decreases by 0.08% for every 1g/dL increase in Hb.

Citation: Bai XF, Wang H, Zhao QL. Hemoglobin within normal range is negatively related to hemoglobin A1c in a nondiabetic American population aged 16 years and older. *World J Diabetes* 2022; 13(3): 251-259
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/251.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.251

INTRODUCTION

Diabetes mellitus (DM) has a high global incidence. The prevalence and incidence of DM continue to increase annually. DM is a major cause of global morbidity and mortality, and was one of the major causes of death in the United States in 2015. Over 30 million and 86 million Americans suffer from diabetes and prediabetes, respectively, which could increase the occurrence rate of many chronic diseases, especially type 2 DM (T2DM)[1]. Obesity may serve as a major inducement factor for diabetes, and the prevalence of diabetes and obesity are increasing[2]. Diabetes status can be classified into three categories: nondiabetes, prediabetes, and diabetes (T2DM)[3]. Chronic prediabetes and diabetes often cause a series of complications, including renal, ophthalmological, neurological, and vascular complications. It is well known that controlling high blood glucose levels could reduce and postpone the appearance and progression of DM-related complications[4]. Therefore, many prospective ongoing clinical studies are evaluating the efficacy of new and rarely studied diabetes biomarkers.

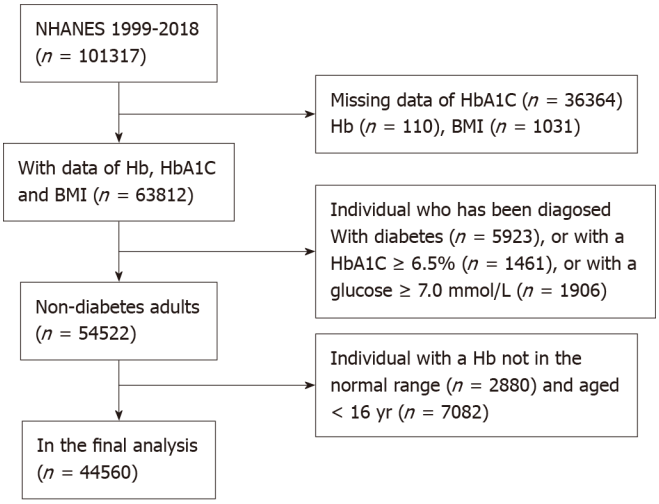
Hemoglobin (Hb) is a protein molecule that only exists in red blood cells (RBCs) that can bind oxygen. In the bloodstream, Hb is glycated. Hemoglobin A1c (HbA1c) acts as glycosylated hemoglobin (GHb) constructed by the nonenzymatic binding of glucose to valine at the N-terminus of the Hb β chain, which is the most abundant and common Hb in human erythrocytes. The GHb (HbA1c) level represents the percentage of Hb proteins bound to glucose. Glycemic control has been assessed using GHb. The higher the primary environmental level of blood glucose, the higher the HbA1c level[5]. However, the relationship between the Hb and HbA1c levels remains unclear. Hence, our study aimed to reveal the relationship between the normal level of Hb and GHb in a nondiabetic American population aged ≥ 16 years through cross-sectional investigation data obtained from the 1999-2018 National Health and Nutrition Examination Survey (NHANES).

MATERIALS AND METHODS

Population research

This study analyzed the NHANES data from 1999 to 2018 (20 years). The NHANES participants are representative of the non-institutionalized civilians in America employed by the NHANES multistage stratified sampling design[6].

A total of 101317 participants were registered in the NHANES 1999-2018 database. In this research, 44560 adults aged ≥ 16 years with Hb and HbA1c level data were considered available. We excluded the individual cases with missing HbA1c data (n = 36364); with missing Hb data (n = 110); with missing body mass index (BMI) data (n = 1031); aged < 16 years (n = 7,082); diagnosed with diabetes (n = 5923); with an HbA1c level of > 6.5% (n = 1461) or a glucose level of > 7.0 mol/L (n = 1906); and with an abnormal Hb level (n = 2880). Furthermore, 44560 nondiabetic patients were included in the final analysis (Figure 1).



DOI: 10.4239/wjd.v13.i3.251

Figure 1 Flowchart of study participants. NHANES: National Health and Nutrition Examination Survey; HbA1C: Hemoglobin A1c; BMI: Body mass index.

Variable research

The exposure variable was the Hb level. The method used to derive the complete blood count (CBC) parameters was based on the Beckmann Kurt counting and grading method, combined with an automatic dilution and hybrid device used for sample treatment and a single beam photometer for the determination of the Hb level.

The outcome variable was the HbA1c level. The HbA1c whole blood sample was processed, stockpiled, and transferred to the University of Kansas, Columbia, Missouri.

The multivariate model contained variables that may confuse the association between the HbA1c and Hb. Age; sex; ethnicity; education level; marital status; smoking behavior; BMI; levels of blood glucose, uric acid, total protein, alanine aminotransferase, cholesterol, and serum creatinine; platelet count; and white blood cell and RBC counts were acquired from questionnaires. Smoking behavior was derived from the question "Have you/Has SP smoked more than 100 cigarettes in your/his/her whole life?" The variable name was SMQ020. The SAS label referred to having smoked more than 100 cigarettes in a lifetime. Marital status was defined as follows: unmarried (never married), married (including married and living with partner), divorced (including widowed, divorced, and separated), and unknown (including refused, unknown, and missing). Educational level was quantified as follows: less than high school (including less than the 9th grade and 9th to 11th grade), high school, and above high school (including some university or AA degrees and above university). Race was classified as follows: Mexican Americans, non-Hispanic whites, non-Hispanic blacks, Hispanics, and others, including multiple ethnic groups. BMI was measured at the mobile examination center and calculated as weight/height^2. Blood glucose, total protein, uric acid, cholesterol, alanine aminotransferase, and serum creatinine levels and other data were obtained through the Beckman Synchron LX20 standard biochemical curve analysis. The platelet and white blood cell/RBC count data were obtained from a CBC with a 5-part differential. Diabetes was defined as an HbA1c level of > 6.5% or a fasting blood glucose level of > 7 mmol/L, according to the 2019 American Diabetes Association standards[7]. Details of the data are presented in Table 1 and freely available on the NHANES website (www.cdc.gov/nchs/nhanes/).

Further subgroup analyses were performed based on sex and age. The quartile classification of the Hb level and sensitivity analysis was performed, and the trend P value was calculated. This was followed by a subgroup analysis sorted by age and sex. We applied smoothing curve fitting and generalized additive models to search for the potential nonlinear relationship between the GHb and Hb levels. Piecewise linear regression was employed to analyze the threshold effect of HbA1c. All analyses incorporated the NHANES sampling weights. Statistical significance was set at $P < 0.05$.

RESULTS

A total of 44560 participants ≥ 16 years of age were included in this study. The weighted distributions of sex are shown in Table 1. Women were more educated than men and comprised a higher percentage of non-Hispanic whites and older age individuals; they also had higher cholesterol levels and white blood cell and platelet counts, lower percentage of smoking at least 100 cigarettes in a lifetime, lower levels of alanine aminotransferase, total protein, serum uric acid, serum creatinine, blood glucose, Hb, and HbA1c, and a lower RBC count.

Table 1 Characteristics of participants in the present work

	Male (*n* = 22255)	Female (*n* = 22305)	*P* value
Age (yr)	42.12 ± 16.96	43.92 ± 17.71	< 0.0001
Race/ethnicity (%)			< 0.0001
Non-Hispanic White	68.87	70.50	
Non-Hispanic Black	9.90	9.51	
Mexican American	9.07	7.61	
Other race/ethnicity	12.16	12.38	
Education level (%)			< 0.0001
Less than high school	15.25	13.56	
High school	22.89	21.07	
More than high school	53.71	58.36	
Others	8.15	7.01	
Marital status			< 0.0001
Never married	22.22	17.57	
Married	61.53	57.48	
Divorced	10.22	19.85	
Others	6.03	5.10	
Smoked at least 100 cigarettes in life (%)			< 0.0001
Yes	48.27	37.03	
No	44.76	56.91	
Others	6.97	6.06	
Body mass index (kg/m^2)	27.91 ± 5.70	27.95 ± 7.01	0.4535
Alanine aminotransferase (U/L)	29.29 ± 22.06	20.74 ± 20.30	< 0.0001
Serum creatinine (μmol/L)	85.93 ± 26.19	66.31 ± 18.96	< 0.0001
Blood glucose (mmol/L)	5.08 ± 0.61	4.95 ± 0.59	< 0.0001
Total protein (g/L)	72.34 ± 4.53	71.24 ± 4.62	< 0.0001
Uric acid (μmol/L)	361.26 ± 72.02	277.45 ± 68.64	< 0.0001
Cholesterol (mmol/L)	5.0 ± 1.1	5.1 ± 1.1	< 0.0001
White blood cell count (10^9/L)	7.10 ± 2.30	7.30 ± 2.21	< 0.0001
Red blood cell count (10^{12}/L)	4.99 ± 0.41	4.48 ± 0.36	< 0.0001
Platelet count (10^9/L)	240.75 ± 57.14	266.28 ± 64.99	< 0.0001
Hemoglobin (g/dL)	15.28 ± 1.02	13.64 ± 0.93	< 0.0001
Hemoglobin A1c (%)	5.34 ± 0.36	5.32 ± 0.37	< 0.0001

Data are show in mean ± SD, including age, body mass index, alanine aminotransferase, cholesterol, creatinine, blood glucose, red/white blood cell count, platelet count, total protein, serum uric acid, hemoglobin, and hemoglobin A1c. Weighted linear regression model was employed to compute the *P* value. Categorical variables are represented in percentage (%), including race, educational level, marital status, smoking (> 100 cigarettes in life), while weighted chi-square test was employed to compute the *P* value.

The correlation between Hb and HbA1c obtained by multiple regression analysis is shown in Table 2. There was a negative correlation between the Hb and HbA1c levels (β = -0.01; 95%CI: -0.01, -0.01) in the unadjusted model. The correlation remained significant with the smallest adjustment for age and sex in the regression model (β = -0.01; 95%CI: -0.01, -0.00). After further adjusting the covariates with the estimated impact of the HbA1c level in the model exceeding 10%, the correlation remained negative (β = -0.08; 95%CI: -0.08, -0.07). *P* value was < 0.001 for trend.

Table 2 Relation between hemoglobin (1 g/dL) and hemoglobin A1c level (%)

	Unadjusted model β (95%CI)	Minimally adjusted model β (95%CI)	Fully adjusted model β (95%CI)
Hemoglobin	-0.01 (-0.01, -0.01)[c]	-0.01 (-0.01, -0.00)[c]	-0.08 (-0.08, -0.07)[c]
Hemoglobin (Quartile)			
Q1	Reference	Reference	Reference
Q2	0.01 (0.00, 0.02)	0.01 (-0.00, 0.01)	-0.06 (-0.07, -0.05)[c]
Q3	0.02 (0.01, 0.03)[b]	0.01 (-0.00, 0.02)	-0.12 (-0.13, -0.11)[c]
Q4	-0.04 (-0.05, -0.03)[c]	-0.02 (-0.03, -0.01)[c]	-0.23 (-0.24, -0.22)[c]
P for trend	< 0.001	< 0.001	< 0.001

[a]*P*< 0.01.
[b]*P*< 0.001.
[c]*P*< 0.0001.
Three models were employed to analyze the relation in this work, namely, unadjusted model, minimally adjusted model, and fully adjusted model. No covariates were regulated in unadjusted model. Only age and gender were regulated in minimally adjusted model. Lastly, in fully adjusted model, all parameters were adjusted, including age, gender, ethnicity, educational level, marital status, smoking behavior, body mass index, uric acid, total protein, serum creatinine, blood glucose, alanine aminotransferase, cholesterol, platelet count, and red/ white blood cell count.

The correlation was still negative in the subgroup analysis classified by age (16-29 years, β = -0.011; 95%CI: -0.015, -0.008; 30-51 years, β = -0.004; 95%CI: 0.008, 0.001; 52-85 years, β = -0.021; 95%CI: -0.025, -0.016) and sex (men, β = -0.057; 95%CI: -0.062, -0.052; women, β = -0.012; 95%CI: -0.017, -0.007) when the covariates were controlled. The results are presented in Table 3. The smooth curve fitting and generalized additive model further verified the negative correlation between the Hb and HbA1c levels (Figures 2-4).

A nonlinear relationship between Hb and HbA1c was observed when the Hb levels reached the turning point (13.2 g/dL) [adjusted odds ratio (OR), -0.04; 95%CI: -0.05, -0.03; $P < 0.0001$) and when the Hb levels exceeded 13.2 g/dL (adjusted OR, -0.10; 95%CI: -0.10, -0.09; $P < 0.0001$). The results are presented in Table 4.

DISCUSSION

In the present study, we examined numerous samples of American nondiabetic individuals aged ≥ 16 years to investigate the relationship between the Hb and HbA1c levels in the normal range. Our study showed that the Hb and HbA1c levels were negatively correlated in both men and women.

HbA1c is a GHb, which is a nonenzymatic reaction of glucose binding to Hb. HbA1c is considered as a better marker to evaluate the state of DM compared with blood glucose monitoring, and it is stable and able to represent the average blood glucose level over the past 2-3 mo. The HbA1c levels are affected by a large number of factors, such as race, RBC disorders, and hemoglobinopathies.

A large retrospective cohort study conducted by Grossman *et al* included 11,352 individuals without diabetes and assessed the correlation between the Hb and HbA1c levels. The fifth highest Hb level of HbA1c individuals was significantly lower than that of the other fifth of individuals, and the correlation between the Hb and HbA1c levels was negligible[8]. However, Lai *et al*[9] found that in 1659 Chinese nondiabetic adults aged 20-49 years, the normal Hb levels were negatively correlated with HbA1c.

In our study, we found that the correlation between the Hb and HbA1c levels was obviously negative in the unadjusted model. When minimal adjustments to sex and age were made in the regression model and when the model further adjusted for the estimated value of the HbA1c level to exceed 10 covariates, the association was still significant. Our results were generally consistent with those of Lai *et al*[9]'s studies on Chinese populations.

Since 1999, the NHANES has investigated approximately 5000 people in 15 different counties in America every year[8,10-13]. Each participant represents approximately 65000 people across the country, and such individuals have made significant contributions to the study. Our research had a large-scale, population-based research design; therefore, our results can be extended to the entire American population. However, there are some limitations that should be noted. First, a cause-and-effect relationship between Hb and HbA1c levels could not be established as a consequence of the cross-sectional design of our research. Longitudinal, prospective, large-scale human studies are needed at this point. Second, some covariant data were extracted from self-reporting, which may be easily affected by self-reporting bias. Nevertheless, the data were gathered by skilled interviewers in accordance with

Table 3 Age- and sex-stratified analysis of correlation for hemoglobin (g/dL) and hemoglobin A1c (%)

	Unadjusted model β (95%CI)	Minimally adjusted model β (95%CI)	Fully adjusted model β (95%CI)
Stratified by age			
16-29	-0.011 (-0.015, -0.008)[a]	-0.054 (-0.059, -0.048)[a]	-0.104 (-0.110, -0.097)[a]
30-51	-0.004 (-0.008, 0.001)	-0.037 (-0.042, -0.031)[a]	-0.090 (-0.097, -0.083)[a]
52-85	-0.021 (-0.025, -0.016)[a]	-0.023 (-0.028, -0.017)[a]	-0.084 (-0.091, -0.077)[a]
Stratified by sex			
Men	-0.057 (-0.062, -0.052)[a]	-0.031 (-0.036, -0.027)[a]	-0.085 (-0.091, -0.080)[a]
Women	-0.012 (-0.017, -0.007)[a]	-0.033 (-0.038, -0.029)[a]	-0.091 (-0.096, -0.085)[a]

[a]$P < 0.0001$.

Three models were employed to analyze the relation in this work, namely, unadjusted model, minimally adjusted model, and fully adjusted model. No covariates were regulated in unadjusted model. Only age was regulated in minimally adjusted model. Lastly, in fully adjusted model, all parameters were regulated, including age, gender, ethnicity, educational level, marital status, smoking behavior, body mass index, uric acid, total protein, serum creatinine, blood glucose, alanine aminotransferase, cholesterol, platelet count, and red/ white blood cell count.

Table 4 Threshold effect analysis of hemoglobin-on-hemoglobin A1c using piecewise linear regression

Point of hemoglobin (g/dL)	Odd ratio (95%CI)	*P* value
< 13.2	-0.04 (-0.05, -0.03)	< 0.0001
> 13.2	-0.10 (-0.10, -0.09)	< 0.0001

A threshold of 13.2g/dL for the hemoglobin existed for hemoglobin A1c. Parameters were adjusted, including age, race, body mass index, smoking (> 100 cigarettes in life), educational level, marital status, serum uric acid, alanine aminotransferase, creatinine, blood glucose, total protein, cholesterol, red/white blood cell count, and platelet count.

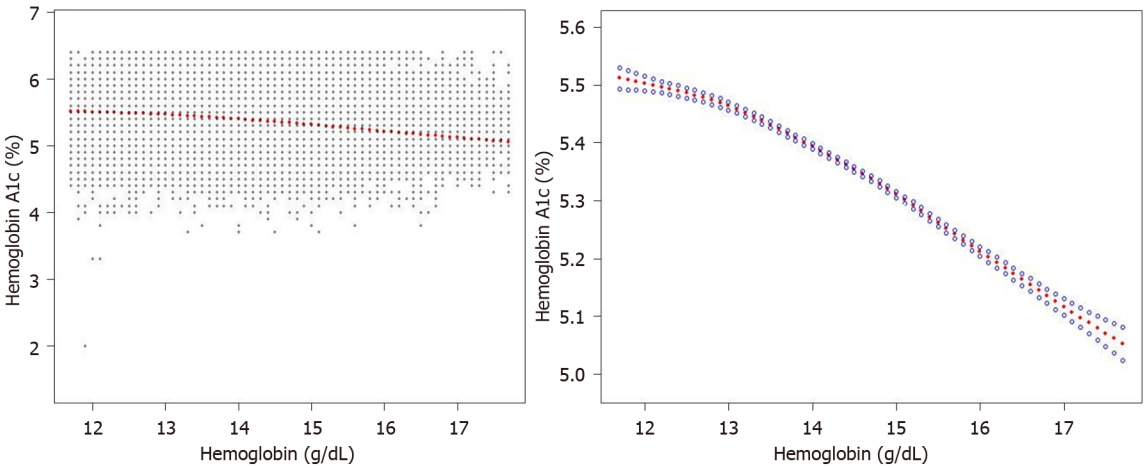


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Figure 2 Relation between hemoglobin and hemoglobin A1c among participants. Samples are shown in black points and smooth curve fitting data points is represented in solid red line. Besides, the 95%CI is represented in blue band. All parameters were modified, including age, gender, ethnicity, educational level, marital status, blood glucose, smoke behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count, and red/white blood cell count.

standardized agreements. Third, we excluded individuals with diabetes and abnormal Hb levels and those younger than 16 years of age. Therefore, our conclusions do not apply to these groups of people. Fourth, although several potential confounding factors were regulated, other potential confounding factors were not included in this study. Therefore, our study may include biases. Further prospective studies with large sample sizes are needed which include the measurement of these additional variables.

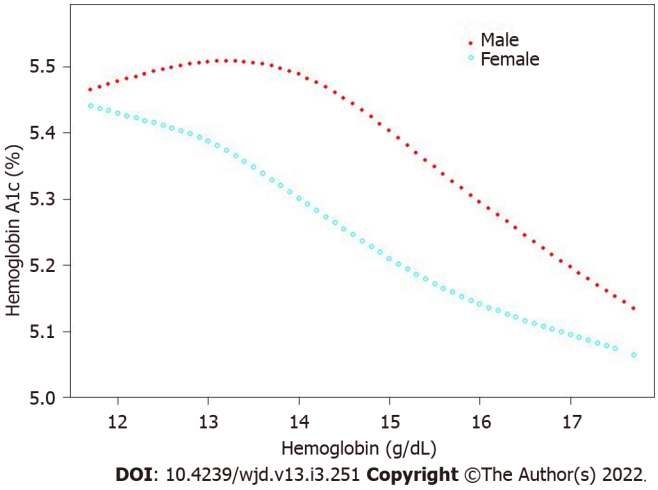

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Figure 3 Sex-stratified analysis of correlation for hemoglobin and hemoglobin A1c. All parameters were regulated, including age, ethnicity, educational level, marital status, blood glucose, smoking behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count and red/white blood cell count.

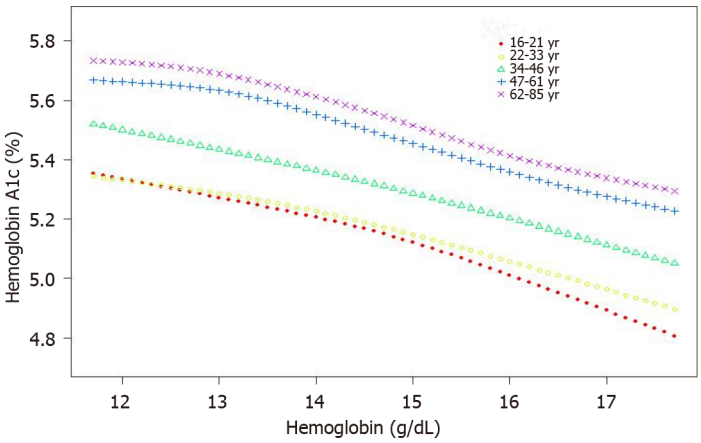

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Figure 4 Age-stratified analysis of correlation for hemoglobin and hemoglobin A1c in five groups. All parameters were adjusted, including sex, race, educational level, marital status, blood glucose, smoking behavior, body mass index, uric acid, total protein, alanine aminotransferase, cholesterol, serum creatinine, platelet count, and red/white blood cell count.

CONCLUSION

In conclusion, our results show that in the nondiabetic American population aged ≥ 16 years, the Hb levels were negatively correlated with the HbA1c levels within the normal range in both men and women. The Hb levels were independent and negatively related to the HbA1c levels.

ARTICLE HIGHLIGHTS

Research background

The relationship between hemoglobin (Hb) and hemoglobin A1c (HbA1c) remains unclear.

Research motivation

To elucidate the relationship between Hb and HbA1c in a nondiabetic population aged ≥ 16 years in America.

Research objectives

To elucidate the relationship between Hb and HbA1c.

Research methods
The relationship was estimated using a multivariate regression.

Research results
Hb levels are negatively correlated with HbA1c.

Research conclusions
Normal Hb levels are negatively correlated with HbA1c in nondiabetic Americans aged ≥ 16 years.

Research perspectives
From a clinical point of view, HbA1c decreases by 0.08% for every 1 g/dL increase in Hb.

FOOTNOTES

Author contributions: Bai XF sorted out the data and wrote the draft; Wang H and Zhao QL revised the article.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: The data that support the findings of this study are openly available by contacting the corresponding author.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 260-271

DOI: 10.4239/wjd.v13.i3.260

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Observational Study

Age at diagnosis of type 2 diabetes and cardiovascular risk factor profile: A pooled analysis

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Jialal I

Received: May 28, 2021
Peer-review started: May 28, 2021
First decision: June 24, 2021
Revised: July 8, 2021
Accepted: February 10, 2022
Article in press: February 10, 2022
Published online: March 15, 2022

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Abstract

BACKGROUND

The diagnosis of type 2 diabetes (T2D) in younger adults, an increasingly common public health issue, is associated with a higher risk of cardiovascular complications and mortality, which may be due to a more adverse cardiovascular risk profile in individuals diagnosed at a younger age.

AIM

To investigate the association between age at diagnosis and the cardiovascular risk profile in adults with T2D.

METHODS

A pooled dataset was used, comprised of data from five previous studies of adults with T2D, including 1409 participants of whom 196 were diagnosed with T2D under the age of 40 years. Anthropometric and blood biomarker measurements included body weight, body mass index (BMI), waist circumference, body fat percentage, glycaemic control (HbA1c), lipid profile and blood pressure. Univariable and multivariable linear regression models, adjusted for diabetes duration, sex, ethnicity and smoking status, were used to investigate the association between age at diagnosis and each cardiovascular risk factor.

RESULTS

A higher proportion of participants diagnosed with T2D under the age of 40 were female, current smokers and treated with glucose-lowering medications, compared to participants diagnosed later in life. Participants diagnosed with T2D under the age of 40 also had higher body weight, BMI, waist circumference and body fat percentage, in addition to a more adverse lipid profile, compared to participants diagnosed at an older age. Modelling results showed that each one year reduction in age at diagnosis was significantly associated with 0.67 kg higher body weight [95% confidence interval (CI): 0.52-0.82 kg], 0.18 kg/m^2 higher BMI (95%CI: 0.10-0.25) and 0.32 cm higher waist circumference (95%CI: 0.14-0.49), after adjustment for duration of diabetes and other confounders. Younger age at diagnosis was also significantly associated with higher HbA1c, total cholesterol, low-density lipoprotein cholesterol and triglycerides.

CONCLUSION

The diagnosis of T2D earlier in life is associated with a worse cardiovascular risk factor profile, compared to those diagnosed later in life.

Key Words: Type 2 diabetes mellitus; Early-onset adult type 2 diabetes; Age of onset; Cardiovascular risk; Young adults; Glycaemic control; Obesity

Core Tip: The diagnosis of type 2 diabetes (T2D) in younger adults, an increasingly common public health issue, is associated with a higher risk of cardiovascular complications and mortality, which may be due to a more adverse cardiovascular risk profile in individuals diagnosed at a younger age. This analysis demonstrates the adverse effect of younger diagnosis of T2D on cardiovascular risk factors, highlighting the need for targeted multifactorial age-appropriate interventions in order to improve the cardiovascular risk factor profile of younger adults with T2D and reduce their subsequent risk of cardiovascular complications and mortality.

Citation: Barker MM, Zaccardi F, Brady EM, Gulsin GS, Hall AP, Henson J, Htike ZZ, Khunti K, McCann GP, Redman EL, Webb DR, Wilmot EG, Yates T, Yeo J, Davies MJ, Sargeant JA. Age at diagnosis of type 2 diabetes and cardiovascular risk factor profile: A pooled analysis. *World J Diabetes* 2022; 13(3): 260-271
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/260.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.260

INTRODUCTION

Type 2 diabetes (T2D), a significant and increasing public health issue, was tradi-tionally considered a disease of mid- to late adulthood[1]. However, the prevalence of T2D among younger adults (*e.g.*, diagnosed < 40 years of age; "early-onset adult T2D") has rapidly increased over the last few decades, now constituting between 15%-20% of all adults with T2D worldwide[2-4].The diagnosis of T2D at an earlier age is associated with an increased relative risk of mortality and of both microvascular and macrovascular complications, as highlighted by a recent meta-analysis of 26 studies[5]. Previous studies have suggested that a more adverse cardiovascular risk profile in adults diagnosed with T2D at a younger age [including higher glycaemic control (HbA1c), higher prevalence of obesity and a worse lipid profile] may explain some of the increased risk of mortality and complications observed among younger adults with T2D[3,6-9]. However, some conflicting results emerged within these studies[3,6,8,

9], whilst most have investigated the effect of age at diagnosis as a categorical variable (early- *vs* later-onset T2D). Consequently, estimates for the difference in risk factors incurred by each one year reduction in age at diagnosis are sparse[3].

Given the increase in prevalence of early-onset T2D and the higher risk of mortality and cardiovascular complications observed in younger adults with T2D, a comprehensive understanding of the effect of diagnostic age on cardiovascular risk factor profile is crucial. This analysis aimed to investigate the association between age at diagnosis, as a continuous variable, and the cardiovascular risk profile of adults with T2D, including measures of adiposity, HbA1c, lipid metabolism and blood pressure, using a pooled dataset of research trial data from multi-ethnic study populations in the United Kingdom.

MATERIALS AND METHODS

Pooled dataset

This analysis used a pooled dataset, comprising data from five previous or ongoing studies of adults with T2D in the United Kingdom: Chronotype of Patients with Type 2 Diabetes and Effect on Glycaemic Control (CODEC)[10], Effects of Liraglutide in Young Adults With Type 2 Diabetes (LYDIA), Early Detection of Cardiac Dysfunction and Health Behaviours in the Young with Type 2 Diabetes (EXPEDITION)[11], Diabetes Interventional Assessment of Slimming or Training to Lessen Inconspicuous Cardiovascular Dysfunction (DIASTOLIC)[12] and Prevalence and Determinants of Subclinical Cardiovascular Dysfunction in Adults with Type 2 Diabetes Mellitus (PREDICT)[13]. The rationale, design and eligibility criteria of these studies have been published previously, in addition to the main outcomes of the completed trials (LYDIA, EXPEDITION, DIASTOLIC)[10-14]. The aims, eligible age ranges and progress of each study are described in Table 1. Each study received ethical approval and all participants provided written informed consent. The pooled dataset used in the current analysis included all participants diagnosed at 16 years or older.

Outcome measurement

Outcome data used in this analysis were collected during baseline assessments within the pooled studies. During these baseline visits, information on demographics (including current age at visit), medical history (including age at T2D diagnosis) and medication use were collected. Anthropometric [including body weight, body mass index (BMI), waist circumference, and body fat percentage] and blood pressure measurements were collected using standardised procedures, and a blood sample was taken for measurement of routine circulating biomarkers (performed by accredited NHS clinical pathology laboratories using quality controlled enzymatic assays).

Statistical analysis

In order to compare demographic variables, cardiovascular complications, medication use, and cardiovascular risk factors [body weight, BMI, waist circumference, body fat percentage, HbA1c, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, systolic and diastolic blood pressure] by age at diagnosis, all data were first presented as median [interquartile range (IQR)] or percentages, as appropriate, using three diagnostic age categories: Diagnosed under 40 years of age, diagnosed between 40-59 years and diagnosed aged 60 years or older. Linear regression models were then used to investigate the association of age at diagnosis, used as a continuous variable, and each cardiova-scular risk factor. In order to assess the possibility of deviations from linearity, models were also conducted using a spline transformation of age at diagnosis for each cardiovascular risk factor. These models were compared to the linear models using Bayesian Information Criterion scores. For all models, there was no evidence of a significant difference between the spline and the linear models, therefore linear regression was used for the analyses.

As younger diagnosis may often predispose individuals to longer duration of T2D, it was important to assess whether any association between age at diagnosis and cardiovascular risk factors remained once diabetes duration was controlled for, as well as after adjustment for other important confounding variables. Therefore, three models were constructed for each cardiovascular risk factor: Model 1 (unadjusted univariable model), Model 2 (adjusted for duration of T2D alone), Model 3 (adjusted for duration of T2D, sex, ethnicity and smoking status). Robust standard errors were used to account for the clustering of data from the different studies.

RESULTS

Participant characteristics

In total, 1409 participants were included in the pooled dataset, of whom 196 (13.9%) were diagnosed with T2D under the age of 40 years, 846 (60.0%) were diagnosed between 40-59 years, and 367 (26.1%)

Table 1 Summary of studies included in the pooled dataset

Study name	Aim	Eligible age range (yr)	Exclusion criteria[1]	Clinical Trials.gov Registration Number	Ongoing/completed
CODEC	Observational study to investigate the effect of chronotype on glycaemic controls in adults with T2DM	18-75	N/A	NCT02973412	Ongoing
LYDIA	Randomised active-comparator trial to investigate the effect of liraglutide compared to sitagliptin on cardiac structure and function in younger adults with T2DM	18-60	Treatment with insulin, SGLT-2 inhibitors, GLP-1 receptor agonists of DPP-4 inhibitors; Active cardiovascular disease, including history of myocardial infarction within the past 6 mo and/or heart failure	NCT02043054	Completed
EXPEDITION	Observational study to phenotype younger adults with T2DM	18-40	N/A	N/A	Completed
DIASTOLIC	Randomised controlled trial to compare diet and exercise interventions to standard care in adults with T2DM	18-65	Current treatment with more than three glucose-lowering medications or insulin; Stroke, peripheral vascular disease, atrial fibrillation, heart failure/disease, angina	NCT02590822	Completed
PREDICT	Observational study to investigate the prevalence and determinants of subclinical cardiovascular dysfunction in adults with T2DM	18-75	Stroke, symptomatic peripheral vascular disease, atrial fibrillation, history of heart failure, history of myocardial infarction, moderate or severe heart valve disease, angina	NCT03132129	Ongoing

[1]Criteria related to cardiovascular complications or medications relevant to this analysis.

T2DM: Type 2 diabetes mellitus; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; DPP-4: Dipeptidyl peptidase-4.

were diagnosed aged 60 years or older. The age at which participants were diagnosed with T2D ranged from 18 to 74 years (Figure 1). Table 2 presents participant characteristics by diagnostic age categories. Participants who were diagnosed under 40 years had a median current age of 46 years (IQR: 38-55) at study entry compared to 71 years (IQR: 68-73) among participants diagnosed at 60 years or over. As expected, median T2D duration was highest among participants who were diagnosed under the age of 40 years (11 years, IQR: 5-21) and lowest in parti-cipants diagnosed at 60 years or over (5 years, IQR: 3-8 years).

A higher proportion of participants diagnosed under the age of 40 were female (49.0%) compared to those diagnosed between 40-59 years (33.2%) or over 60 years (33.8%). Although the most common ethnicity across all diagnostic age groups was white, there were proportionally more Asian participants in those diagnosed before the age of 40 (28.1%), compared to those diagnosed aged 40-59 years or aged 60 years or older (17.1% and 7.6%, respectively). Participants diagnosed under the age of 40 were also more likely to be current smokers (12.2%) and to have a family history of T2D (45.9%). The prevalence of all cardiovascular complications was lower at the point of study entry among participants diagnosed under the age of 40, compared to those diagnosed at the age of 40 or over. The prevalence of metabolic syndrome was higher among participants diagnosed under 40 years (94.1%) compared to those diagnosed between 40-59 years (90.2%) or at 60 years or over (85.6%). The proportion of participants using glucose-lowering medications was higher in participants diagnosed before 40 years (94.9%) compared to participants diagnosed between 40-59 years (89.5%) or 60 years or over (70.0%), whilst the opposite trend was observed for lipid-lowering or antihypertensive medications.

Cardiovascular risk factors

Table 3 displays participants' cardiovascular risk factor profiles by diagnostic age categories. Participants diagnosed with T2D under the age of 40 had a higher body weight (95.2 kg, IQR: 82.5-108.9 kg) compared to participants diagnosed between the ages of 40-59 years (92.0 kg, IQR: 79.6-105.6 kg) or at 60 years or over (84.6 kg, IQR: 73.7-97.4 kg). BMI was also highest among participants diagnosed under 40 years (33.0 kg/m^2, IQR: 29.0-36.8 kg/m^2) compared to those diagnosed between 40-59 years (31.6 kg/m^2, IQR: 28.0-35.3 kg/m^2) or those diagnosed later than 60 years of age (29.2 kg/m^2, IQR: 26.3-33.0 kg/m^2). A similar trend was observed for waist circumference and body fat percentage.

Median HbA1c was also higher among participants diagnosed under the age of 40 (7.5%, IQR: 6.7%-8.5%) compared to those diagnosed between the age of 40-59 years (7.1%, IQR: 6.4%-7.9%) or at 60 years or over (6.5%, IQR: 6.0%-7.2%). Additionally, a marginally more adverse lipid profile was identified among participants diagnosed under the age of 40, showing higher total cholesterol, LDL cholesterol and triglycerides, and lower HDL cholesterol compared to participants diagnosed at 40 years or over.

Table 2 Demographic characteristics, cardiovascular complications and medication use by age of diagnosis

	Age at T2DM diagnosis			Total sample (*n* = 1409)
	Under 40 yr (*n* = 196)	40-59 yr (*n* = 846)	60 yr or over (*n* = 367)	
Number of participants from each dataset, *n* (%)				
CODEC	111 (56.6)	636 (75.2)	326 (88.8)	1073 (76.2)
LYDIA	35 (17.9)	41 (4.9)	0 (0.0)	76 (5.4)
EXPEDITION	20 (10.2)	0 (0.0)	0 (0.0)	20 (1.4)
DIASTOLIC	17 (8.7)	72 (8.5)	0 (0.0)	89 (6.3)
PREDICT	13 (6.6)	97 (11.5)	41 (11.2)	151 (10.7)
Current age, yr (*n* = 1408)	46 (38-55)	61 (56-67)	71 (68-73)	63 (55-69)
Diabetes duration, yr (*n* = 1408)	11 (5-21)	10 (5-15)	5 (3-8)	8 (4-14)
Sex, *n* (%)				
Male	100 (51.0)	565 (66.8)	243 (66.2)	908 (64.4)
Female	96 (49.0)	281 (33.2)	124 (33.8)	501 (35.6)
Ethnicity, *n* (%)				
White	125 (63.8)	665 (78.6)	333 (90.7)	1123 (79.7)
Asian	55 (28.1)	145 (17.1)	28 (7.6)	228 (16.2)
Other	6 (3.1)	33 (3.9)	5 (1.4)	44 (3.1)
Unknown	10 (5.1)	3 (0.4)	1 (0.3)	14 (1.0)
Smoking status, *n* (%)				
Current smoker	24 (12.2)	66 (7.8)	20 (5.5)	110 (7.98)
Ex-smoker	58 (29.6)	367 (43.4)	182 (49.6)	607 (43.1)
Never smoked	114 (58.2)	413 (48.8)	165 (45.0)	692 (49.1)
Family history of T2D, *n* (%)				
Yes	90 (45.9)	326 (38.5)	131 (35.7)	547 (38.8)
No	37 (18.9)	264 (31.2)	169 (46.1)	470 (33.3)
Unknown	69 (35.2)	256 (30.3)	67 (18.3)	393 (27.9)
Cardiovascular complications, *n* (%)				
Myocardial infarction (*n* = 1233)	7 (4.3)	60 (8.0)	36 (11.1)	103 (8.4)
Heart failure (*n* = 1229)	4 (2.5)	12 (1.6)	9 (2.8)	25 (2.0)
Heart valve disease (*n* = 1228)	3 (1.8)	22 (3.0)	11 (3.4)	36 (2.9)
Atrial fibrillation (*n* = 1223)	2 (1.2)	42 (5.7)	20 (6.2)	64 (5.2)
Peripheral vascular disease (*n* = 1227)	7 (4.4)	43 (5.8)	21 (6.5)	71 (5.8)
Stroke (*n* = 1235)	3 (1.9)	31 (4.1)	24 (7.4)	58 (4.7)
Angina (*n* = 1230)	5 (3.1)	60 (8.1)	33 (10.2)	98 (8.0)
Glucose-lowering medication use, *n* (%)				
Any glucose-lowering medication (*n* = 1403)	185 (94.9)	753 (89.5)	257 (70.0)	1195 (85.2)
Insulin	74 (37.8)	178 (21.0)	24 (6.5)	276 (19.6)
Metformin (*n* = 1407)	157 (80.1)	658 (78.0)	234 (63.8)	1049 (74.6)
Sulphonylurias (*n* = 1407)	36 (18.4)	205 (24.3)	51 (13.9)	292 (20.8)
DPP-4 inhibitors	18 (9.2)	139 (16.4)	39 (10.6)	196 (13.9)
GLP-1 agonists	33 (16.8)	63 (7.5)	9 (2.5)	105 (7.5)
SGLT2 inhibitors (*n* = 1389)	27 (17.0)	89 (11.5)	15 (4.1)	131 (10.1)

Other[1] (*n* = 1390)	3 (1.9)	19 (2.4)	4 (1.1)	26 (2.0)
Lipid-lowering medication use, *n* (%)				
Any lipid-lowering medication (*n* = 1407)	112 (57.4)	583 (69.0)	254 (69.2)	949 (67.5)
Statins (*n* = 1408)	108 (55.4)	580 (68.6)	251 (68.4)	939 (66.7)
Fibrates (*n* = 1407)	10 (5.1)	23 (2.7)	4 (1.1)	37 (2.6)
Antihypertensive medication use, *n* (%)				
Any antihypertensive medication (*n* = 1389)	97 (54.8)	582 (68.8)	252 (68.9)	931 (67.0)
ACE inhibitors (*n* = 1390)	68 (38.4)	356 (42.1)	125 (34.1)	549 (39.5)
Alpha blockers (*n* = 1388)	8 (4.6)	86 (10.2)	53 (14.5)	147 (10.6)
Angiotensin receptor blockers (*n* = 1389)	17 (9.7)	134 (15.8)	61 (16.6)	212 (15.3)
Beta blockers (*n* = 1388)	20 (11.4)	157 (18.6)	70 (19.1)	247 (17.8)
Calcium channel blockers (*n* = 1389)	31 (17.6)	246 (29.1)	103 (28.1)	380 (27.4)
Diuretics (*n* = 1389)	25 (14.2)	122 (14.4)	58 (15.8)	205 (14.8)
Metabolic syndrome prevalence, *n* (%)[2] (*n* = 1290)	159 (94.1)	697 (90.2)	298 (85.6)	1154 (89.5)

[1]Includes alpha glucosidase inhibitors, thiazolidinediones and meglitinides.
[2]Defined using the global definition published by Alberti *et al*[24] (2005).
Data presented as median or frequency (%), as appropriate. T2DM: Type 2 diabetes mellitus; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; DPP-4: Dipeptidyl peptidase-4.

Table 3 Cardiovascular risk factors by age at diagnosis

	Age at T2DM diagnosis			Total sample (*n* = 1409)
	Under 40 yr (*n* = 196)	40-59 yr (*n* = 846)	60 yr or over (*n* = 367)	
Weight (kg)	95.2 (82.5-108.9)	92.0 (79.6-105.6)	84.6 (73.7-97.4)	90.6 (78.2-103.8)
BMI (kg/m^2)	33.0 (29.0-36.8)	31.6 (28.0-35.3)	29.2 (26.3-33.0)	31.1 (27.5-35.0)
Waist circumference (cm), *n* = 1403	112.0 (102.2-119.1)	109.0 (100.0-118.0)	104.0 (96.8-113.5)	108.0 (99.0-117.8)
Body fat (%), *n* = 1076	36.9 (29.5-44.5)	34.0 (27.6-40.7)	32.5 (26.3-41.0)	33.8 (27.4-41.2)
HbA1c (%), *n* = 1370	7.5 (6.7-8.5)	7.1 (6.4-7.9)	6.5 (6.0-7.2)	7.0 (6.3-7.8)
Total cholesterol (mmol/L), *n* = 1352	4.4 (3.8-5.2)	4.2 (3.6-4.9)	4.1 (3.5-4.8)	4.2 (3.6-4.9)
LDL cholesterol (mmol/L), *n* = 1308	2.3 (1.8-2.9)	2.1 (1.6-2.7)	2.1 (1.6-2.6)	2.1 (1.6-2.7)
HDL cholesterol (mmol/L), *n* = 1338	1.1 (1.0-1.4)	1.2 (1.0-1.5)	1.3 (1.1-1.5)	1.2 (1.0-1.5)
Triglycerides (mmol/L), *n* = 1351	1.8 (1.2-2.7)	1.7 (1.2-2.3)	1.5 (1.1-2.1)	1.6 (1.1-2.3)
Systolic blood pressure (mmHg), *n* = 1408	128.0 (119.0-140.0)	135.0 (124.0-146.5)	137.0 (125.5-148.7)	135.0 (123.8-146.0)
Diastolic blood pressure (mmHg), *n* = 1408	83.0 (76.5-90.5)	82.0 (76.0-89.0)	79.5 (73.0-86.5)	81.5 (75.0-88.6)

Data presented as median. LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; T2DM: Type 2 diabetes mellitus.

Model results

As shown in Table 4, younger age at diagnosis of T2D was significantly associated with higher body weight, BMI, waist circumference and HbA1c. Results from Model 3 (adjusted for duration of T2D, sex, ethnicity and smoking status) showed that each one year reduction in age at diagnosis of T2D was significantly associated with 0.67 kg [95% confidence interval (CI): 0.52-0.82 kg] higher body weight, 0.18 kg/m^2 (95%CI: 0.10-0.25 kg/m^2) higher BMI, 0.32 cm (95%CI: 0.14-0.49 cm) higher waist circumference and 0.03% (95%CI: 0.03%-0.04%) higher HbA1c. Similarly, results from Model 3 indicate that each one year reduction in age at diagnosis was significantly associated with 0.01 mmol/L (95%CI: 0.01-0.02 mmol/L) higher total cholesterol, 0.01 mmol/L higher LDL cholesterol (95%CI: 0.01-0.02 mmol/L) and 0.02 mmol/L (95%CI: 0.01-0.03 mmol/L) higher triglycerides, after adjustment for the same covariates. Each one year reduction in diagnostic age was significantly associated with 0.22 mmHg

Table 4 Results from linear regression models investigating the effect of age at diagnosis on each cardiovascular risk factor

	Model 1		Model 2		Model 3	
	Estimate	*n*	Estimate	*n*	Estimate	*n*
Weight (kg)	-0.32 [(-0.51) to (-0.14)][a]	1409	-0.45 [(-0.60) to (-0.31)][a]	1408	-0.67 [(-0.82) to (-0.52)][a]	1394
BMI (kg/m^2)	-0.11 [(-0.19) to (-0.02)][b]	1409	-0.15 [(-0.23) to (-0.07)][a]	1408	-0.18 [(-0.25) to (-0.10)][a]	1394
Waist circumference (cm)	-0.21 [(-0.32) to (-0.09)][a]	1403	-0.23 [(-0.41) to (-0.05)][b]	1402	-0.32 [(-0.49) to (-0.14)][a]	1388
Body fat (%)	-0.10 (-0.80 to 0.60)	1076	-0.16 (-0.85 to 0.53)	1075	-0.11 (-0.43 to 0.20)	1073
HbA1c (%)	-0.04 [(-0.04) to (-0.03)][a]	1370	-0.03 [(-0.04) to (-0.03)][a]	1369	-0.03 [(-0.04) to (-0.03)][a]	1356
Total cholesterol (mmol/L)	-0.01 (-0.01 to 0.00)	1352	-0.02 [(-0.02) to (-0.01)][a]	1351	-0.01 [(-0.02) to (-0.01)][a]	1337
LDL cholesterol (mmol/L)	-0.01 (-0.01 to 0.00)[b]	1308	-0.01 [(-0.02) to (-0.01)][a]	1307	-0.01 [(-0.02) to (-0.01)][a]	1294
HDL cholesterol (mmol/L)	0.00 (0.00 to 0.01)	1338	0.00 (0.00 to 0.01)	1337	0.01 (0.00 to 0.01)	1323
Triglycerides (mmol/L)	-0.01 (-0.03 to 0.00)	1351	-0.02 [(-0.03) to (-0.01)][b]	1350	-0.02 [(-0.03) to (-0.01)][a]	1336
Systolic BP (mmHg)	0.24 (0.14 to 0.35)[a]	1408	0.31 (0.11 to 0.51)[b]	1407	0.24 (0.02 to 0.45)[b]	1393
Diastolic BP (mmHg)	-0.10 (-0.21 to 0.02)	1408	-0.20 [(-0.29) to (-0.12)][a]	1407	-0.22 [(-0.30) to (-0.14)][a]	1393

[a]$P < 0.01$.
[b]$P < 0.05$.
Data presented as coefficient. Model 1: Unadjusted univariable model, Model 2: Adjusted for duration of T2D, Model 3: Adjusted for duration of T2D, sex, ethnicity and smoking status. LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; T2DM: Type 2 diabetes mellitus; HbA1c: Glycaemic control; BP: Blood pressure.

(95%CI: 0.14-0.30 mmHg) higher diastolic blood pressure, but 0.24 mmHg (95%CI: 0.02-0.45 mmHg) lower systolic blood pressure.

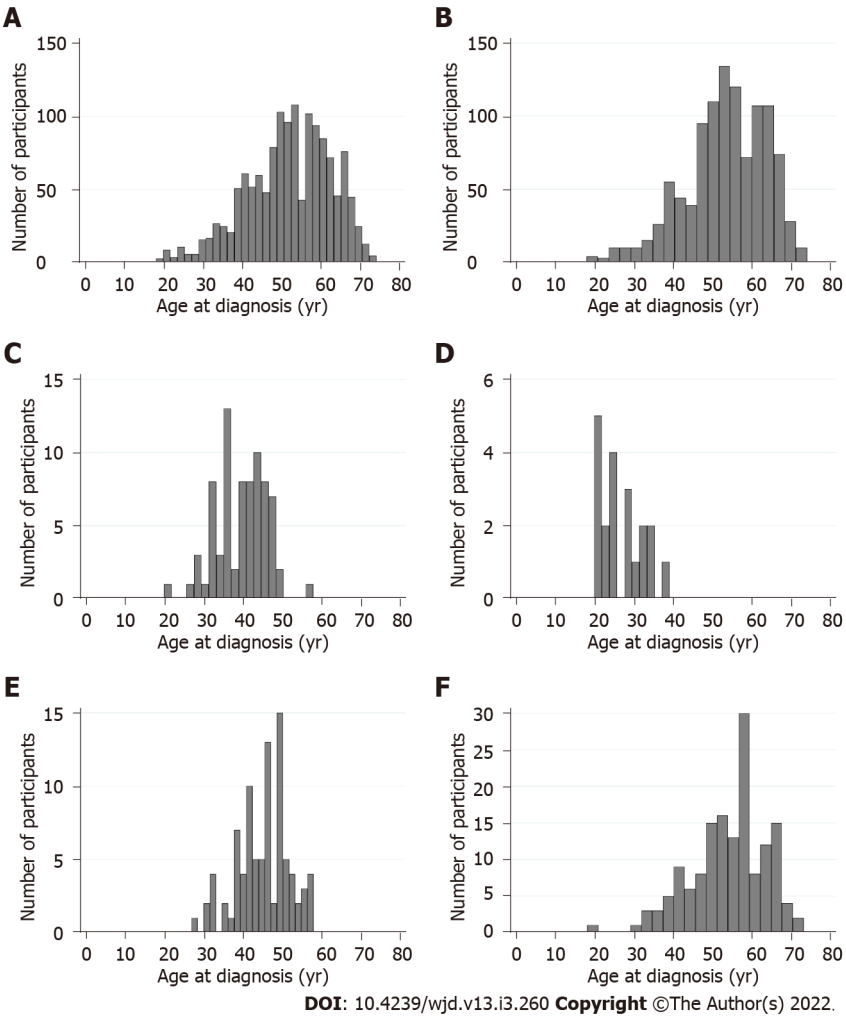
DISCUSSION

This analysis investigated the association between age at diagnosis of T2D and the cardiovascular risk profile among 1410 adults with T2D, using a diverse pooled dataset. The demographic characteristics of the participants included in our analysis varied by diagnostic age, with a higher proportion of females and people of Asian ethnicity among participants diagnosed earlier in life. These results are consistent with previous studies, which have also highlighted increased risk of microvascular and macrovascular complications, as well as incidence of certain co-morbidities, in these high risk subgroups[2,6,15-17]. In our analysis, younger age at diagnosis was also significantly associated with higher BMI, supporting findings from previous studies[3,7,9]. However, the current analysis adds to previous findings by quantifying the change in several cardiovascular risk factors, including body weight, BMI, waist circumference, HbA1c, lipids and blood pressure, for each year earlier diagnosis of T2D.

The association between younger age at diagnosis of T2D and poorer HbA1c identified in this analysis is supported by findings from previous studies[3,6-9]. One study from Asia investigated the association between HbA1c and age of diagnosis, analyzed as a continuous variable, reporting that each one year earlier age at diagnosis was significantly associated with 0.01% higher HbA1c. This is similar, albeit smaller in magnitude, to the results of our current analysis, which identified a 0.03% increase in HbA1c for each year earlier diagnosis.

In the current analysis, diagnosis of T2D at a younger age was significantly associated with higher total and LDL cholesterol and higher triglycerides, however no significant association was observed between age at diagnosis and HDL cholesterol. Similarly, previous studies have reported conflicting results for the relationship between age at diagnosis and lipid profile. For example, Yeung *et al*[3] reported a significant association between age at diagnosis and all lipid markers, whereas Huang *et al*[7] found participants with early-onset T2D had significantly higher total cholesterol and triglycerides compared to those with later-onset T2D, whilst no significant differences were observed from LDL or HDL cholesterol. Younger age at T2D diagnosis was significantly associated with higher diastolic blood pressure and lower systolic blood pressure in this analysis, which is also consistent with previous studies[3,8,9].

The adverse risk factor phenotype observed among younger adults with T2D may contribute to their significantly increased relative risk of microvascular and macrovascular disease and mortality. A recent meta-analysis of 26 studies found that for every one year increase in age at diagnosis, the risk of microvascular disease, macrovascular disease and all-cause mortality fell by 5%, 3% and 4%,



DOI: 10.4239/wjd.v13.i3.260

Figure 1 Frequency distribution of age at diagnosis from each study. A: All participants; B: CODEC participants; C: LYDIA participants; D: EXPEDITION participants; E: DIASTOLIC participants; F: PREDICT participants.

respectively[5]. Research has also shown that the risk of cardiovascular complications and all-cause mortality can be reduced by the control of multiple cardiovascular risk factors, even among younger adults[18-20]. It is therefore imperative that adults with early-onset T2D have access to fit-for-purpose, multifactorial interventions to prevent long term complications, particularly given the global increase in the prevalence of early-onset T2D, and evidence that less than half of younger adults with T2D meet HbA1c targets[21]. Such interventions must also be tailored specifically to the co-occurring challenges that adults with early-onset T2D may face (*e.g.*, early careers, ongoing education and young families), which may be different to older adults, and also allow for sex and cultural differences between individuals. Work to guide the development of such tailored approaches is urgently required, particularly given that adults with early-onset T2D are underrepresented in existing T2D trials[2,22].

This analysis has many strengths. Firstly, the pooled dataset used included a large sample of adults diagnosed with T2D over a wide age range (18-74 years of age) increasing the reliability and generalisability of the conclusions made. The use of age at diagnosis as a continuous variable in the analysis is another benefit, given that most previous literature has investigated age at diagnosis as a categorical variable, comparing people with 'early-onset' T2D to those with 'later-onset' T2D. Although such studies are valuable in assessing whether adults classified as 'early onset' have more cardiovascular risk factors, the range of ages included in the 'early-onset' and 'late-onset' categories are very wide and therefore it was previously unknown how diagnostic age was associated with cardiovascular risk factors within each of these categories. The current study has provided such insight by showing that each reduction of one year in age at diagnosis was significantly associated with a more adverse adiposity, HbA1c, and lipid profile, even after the adjustment for disease duration.

Limitations of the study must also be noted. As the information relating to diagnostic age was self-reported, some participants may not have accurately recalled the date at which they were diagnosed. However, there is evidence that self-reported age at diagnosis of T2D is fairly accurate and a valid measure of diagnostic age[23]. There is also the possibility of selection bias as the data used in this analysis is from volunteers who were motivated to undertake the research studies. In addition, it is

possible that differences in recruitment rates by age at diagnosis and investigated risk factors acted to introduce a form of collider bias. Furthermore, only variables collected in the studies were available for adjustment in this analysis, therefore the effects of other covariates could not be assessed. As the cardiovascular risk factors investigated in this analysis were collected at study enrolment rather than at diagnosis of T2D, the results from this analysis do not indicate how the cardiovascular risk profile differs by diagnostic age at the time of diagnosis. Lastly, the age at diagnosis, age at enrolment and duration of diabetes are correlated and therefore disentangling the effect of one from the others is complex. Nevertheless, the results from the current analysis were unaffected by adjustment for diabetes duration.

CONCLUSION

In conclusion, this study supports previous literature demonstrating an association between younger diagnosis of T2D and a more adverse cardiovascular risk profile. This highlights the need for interventions targeting multiple risk factors in younger adults with T2D in order to reduce their risk of cardiovascular complications and mortality.

ARTICLE HIGHLIGHTS

Research background

The prevalence of type 2 diabetes (T2D) among younger adults is increasing, and is associated with a higher relative risk of mortality and diabetes-related complications compared to older adults with T2D. This may be due to younger adults with T2D having a more adverse cardiovascular risk factor profile.

Research motivation

Although some research has observed a more adverse cardiovascular risk profile among younger adults with T2D, conflicting findings and methodological limitations have emerged within these studies.

Research objectives

To use a pooled dataset to investigate the association between age at diagnosis (as a continuous variable) and the cardiovascular risk factor profile of adults with T2D.

Research methods

The pooled dataset used for this analysis included 1409 participants, 196 of whom were diagnosed with T2D under the age of 40 years. Descriptive analysis and both univariable and multivariable linear regression models were used to investigate the association between diagnostic age and cardiovascular risk factors [weight, body mass index (BMI), waist circumference, body fat percentage, glycaemic control (HbA1c), lipid profile and blood pressure].

Research results

Results from the analysis revealed that younger age at T2D diagnosis was significantly associated with higher weight, BMI, waist circumference, HbA1c and a more adverse lipid profile, even once confounding factors such as diabetes duration, sex and ethnicity were accounted for.

Research conclusions

This analysis supports previous studies which demonstrate an association between younger age at T2D diagnosis and a worse cardiovascular risk factor profile.

Research perspectives

The results from this analysis highlight the importance of multifactorial interventions targeting multiple risk factors in younger adults with T2D, in order to reduce their risk of mortality and cardiovascular complications.

ACKNOWLEDGEMENTS

The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care. Funding for the studies pooled was provided by the NIHR Leicester Biomedical Research Centre (CODEC), the Medical Research Council through an Interdisciplinary Bridging Award (EXPEDITION), NIHR Career Development Fellowship (DIASTOLIC) and

Research Professorship (PREDICT), and the British Heart Foundation through a Clinical Research Training Fellowship (PREDICT). LYDIA was an investigator-initiated study funded by Novo Nordisk. The authors would also like to thank all participants who contributed to the research studies included in this pooled dataset.

FOOTNOTES

Author contributions: Davies MJ and Sargeant JA generated the study idea; Barker MM, Zaccardi F, Henson J, Yates T and Sargeant JA prepared and conducted the analysis; Barker MM, Zaccardi F, Davies MJ and Sargeant JA interpreted the analysis and drafted the manuscript, with clinical and/or academic input from co-authors; all authors reviewed and approved the final manuscript.

Supported by the National Institute for Health Research (NIHR) under its Programme Grants for Applied Research Programme (NIHR201165), as well as by the NIHR Leicester Biomedical Research Centre and the NIHR Applied Research Collaboration East Midlands.

Institutional review board statement: All studies included in the pooled dataset used for this analysis gained full ethical approval (CODEC: 16/WM/0457; EXPEDITION: 08/H0407/8; LYDIA: 13/WM/0311; DIASTOLIC: 15/WM/0222; PREDICT: 17/WM/0192).

Informed consent statement: All participants included in the studies provided written informed consent.

Conflict-of-interest statement: Barker MM, Zaccardi F, Brady EM, Gulsin GS, Hall AP, Henson J, Htike ZZ, McCann GP, Redman EL, Webb DR and Yeo J report no conflicts of interest. Khunti K has acted as consultant, advisory board member and speaker for Abbott, Amgen, Astrazeneca, Bayer, NAPP, Lilly, Merck Sharp and Dohme, Novartis, Novo Nordisk, Roche, Berlin-Chemie AG/Menarini Group, Sanofi-Aventis, Servier, Boehringer Ingelheim, EACME grants from Boehringer Ingelheim, AstraZeneca, Novartis, Novo Nordisk, Sanofi-Aventis, Lilly, Merck Sharp & Dohme. Yates T and Sargeant JA are supported by the NIHR Leicester BRC and have received project funding in the form an investigator-initiated grant from AstraZeneca. EGW has received personal fees from Abbott Diabetes Care, Dexcom, Eli lilly, Insulet, Medtronic, Novo Nordisk, Sanofi Aventis. Davies MJ has acted as consultant, advisory board member and speaker for Novo Nordisk, Sanofi, Lilly and Boehringer Ingelheim, an advisory board member and speaker for AstraZeneca, an advisory board member for Janssen, Lexicon, Servier and Gilead Sciences Ltd and as a speaker for Napp Pharmaceuticals, Mitsubishi Tanabe Pharma Corporation and Takeda Pharmaceuticals International Inc. She has received grants in support of investigator and investigator initiated trials from Novo Nordisk, Sanofi-Aventis, Lilly, Boehringer Ingelheim, AstraZeneca and Janssen.

Data sharing statement: Data included in this pooled analysis will be made available, after publication, to anyone upon reasonable request to the corresponding author.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: United Kingdom

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 272-274

DOI: 10.4239/wjd.v13.i3.272

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Sodium-glucose co-transporter 2 inhibitors induced euglycemic diabetic ketoacidosis within four days of initiation

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Papazafiropoulou A

Received: September 6, 2021
Peer-review started: September 6, 2021
First decision: December 4, 2021
Revised: December 5, 2021
Accepted: February 10, 2022
Article in press: February 10, 2022
Published online: March 15, 2022

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Abstract

Euglycemic diabetic ketoacidosis (EDKA) is a well-known complication of sodium-glucose co-transporter 2 inhibitors, and many cases with variable onset following the initiation of these agents are reported before, with a median onset of approximately 2 wk. This letter discusses a 45-year-old lady who initially presented with ischemic stroke but developed EDKA 4 d after starting empagliflozin, a rare occurrence. The patient had severe metabolic acidosis that necessitated admission into the intensive care unit. Prompt discontinuation of empagliflozin and DKA management resulted in clinical recovery.

Key Words: Euglycemic diabetic ketoacidosis; Sodium-glucose co-transporter 2 inhibitors; Type 2 diabetes mellitus; Empagliflozin

Core Tip: With a steady surge in the prescription of sodium-glucose co-transporter 2 inhibitors (SGLT2-i) in medical conditions including type 2 diabetes mellitus (DM), type 1 DM, and heart failure, there are increasingly reported cases of euglycemic diabetic ketoacidosis (EDKA) with their use. EDKA in the context of SGLT2-i use is reported in various patients with different precipitating factors, some even with no inciting event. One of the rarely reported inciting events is stroke. Another aspect of SGLT2-i induced EDKA which remains relatively less understood is the time of initiation of the drug to the development of EDKA. In our patient, severe EDKA developed within 4 d of empagliflozin initiation, necessitating intensive care and discontinuation of empagliflozin, resulting in complete recovery regarding the EDKA.

Citation: Razok A, Ata F, Ahmed SMI, Al Mohanadi DHSH. Sodium-glucose co-transporter 2 inhibitors induced euglycemic diabetic ketoacidosis within four days of initiation. *World J Diabetes* 2022; 13(3): 272-274
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/272.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.272

TO THE EDITOR

With great interest, we read the recent article "Euglycemic diabetic ketoacidosis: A missed diagnosis" by Nasa *et al*[1]. The authors have described various factors that can potentiate euglycemic diabetic ketoacidosis (EDKA) in sodium-glucose co-transporter 2 inhibitor (SGLT2-i) use. There is a steady increase in EDKA reports secondary to SGLT2-i. Most of the articles mention a precipitating factor behind the development of EDKA in patients taking SGLT2-i. More extensive studies mention no or unknown precipitating factor in 16%-51% of cases[2,3]. This creates a need to explore a possible direct link of SGLT2-i in the development of EDKA in an otherwise healthy patient with diabetes. Acute vascular events such as stroke are infrequent inciting events for EDKA in the setting of SGLT2-i use.

We recently encountered an interesting case of a patient with type 2 diabetes mellitus (T2DM) who was admitted with acute stroke and developed EDKA within 4 d of initiation of empagliflozin.

A 45-year-old woman presented with sudden onset left arm weakness and slurred speech with facial droop. Magnetic resonance imaging revealed a right basal ganglia acute infarction, in addition to left parietal subcortical microangiopathic changes. The patient had a history of breast carcinoma, treated with mastectomy and maintenance tamoxifen. She also had T2DM and was prescribed sitagliptin/metformin 50/1000 mg two tablets daily. However, the patient was non-compliant with the medication and was not checking her blood sugar regularly.

The patient was started on dual antiplatelet therapy (aspirin 100 mg and clopidogrel 75 mg once daily) after establishing the diagnosis of an acute stroke. Her HbA1c was 14%, confirming a poor control of her diabetes. To manage her poorly controlled diabetes mellitus, sitagliptin/metformin was continued, with the addition of insulin glargine 12 units at bedtime and empagliflozin 10 mg once daily.

Four days later, the patient developed vomiting and generalized fatigue. Arterial blood gas showed severe metabolic acidosis with a pH of 6.9 and bicarbonate level of 3 mEq/L (reference range: 22-26 mEq/L). Serum B-hydroxy butyrate was higher than the reported threshold of 9.60 mmol/L (reference range: 0.03-0.3). Her blood glucose level at the time was 10.3 mmol/L (reference range: 3.3-5.5), and her urine dipstick showed +4 ketone. She was diagnosed with severe EDKA and was shifted to the medical intensive care unit for further management and treatment.

Regular insulin infusion and intravenous fluids were initiated, and a right internal jugular line was inserted for monitoring and resuscitation. Her arterial blood gas was measured every 2 h, and serum ketones were measured daily. Forty-eight hours later, the patient's condition improved, and she started tolerating oral feed. Her ABG results showed significant improvement with the closure of the anion gap (Table 1). She was started on subcutaneous glargine 20 units daily and insulin as part 7 units three times a day.

The patient was consequently shifted back to the care of the general medicine team, where her glycemic control was monitored closely. After ensuring the patient's fitness and stability, she was transferred to a physical and occupational therapy rehabilitation facility.

Our case highlights that in the presence of a precipitating factor, SGLT2-i drugs can cause an early and severe EDKA. We recommend that wherever other choices are available, initiation of SGLT2-i should be delayed until patients are otherwise healthy and not admitted with an acute event. SGLT2-i medications should ideally be started in an outpatient setting, and the patients should be counseled not to rely on blood glucose and seek immediate medical attention when experiencing symptoms of DKA.

Table 1 Laboratory investigations of the patient during euglycemic diabetic ketoacidosis

Investigation	Onset	24 h	48 h	Reference
PH	6.9	7.32	7.42	7.35-7.45
HCO_3 (mmol/L)	3	12.5	20.6	22-29
Glucose (mmol/L)	10.3	10.8	6.4	3.3-5.5
Sodium (mmol/L)	140	137	139	133-146
Potassium (mmol/L)	3.8	3.2	3.4	3.5-5.3
Chloride (mmol/L)	101	111	110	95-108
Anion Gap	36	13.5	8.4	10-12
Lactate (mmol/L)	1.3	0.7	0.9	0.36-1.6
B-hydroxybutyrate (mmol/L)	> 9.60	1.22	0.11	0.03-0.3

FOOTNOTES

Author contributions: Razok A, Ata F, and Ahmed SMI wrote the letter; Al Mohanadi DHSH revised the letter.

Conflict-of-interest statement: The authors have no actual or potential conflict of interest to declare.

Country/Territory of origin: Qatar

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Corresponding Author's Membership in Professional Societies: American College of Physicians, 03770816.

S-Editor: Zhang H
L-Editor: Wang TQ
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 March 15; 13(3): 275-277

DOI: 10.4239/wjd.v13.i3.275

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Free fatty acids, glucose, and insulin in type 2 diabetes mellitus

Rob NM Weijers

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Liu L, Yang L

Received: September 23, 2021
Peer-review started: September 23, 2021
First decision: November 8, 2021
Revised: December 24, 2021
Accepted: February 10, 2022
Article in press: February 10, 2022
Published online: March 15, 2022

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Abstract

Xu *et al* used the HOMA2 model to estimate the β-cell function and insulin resistance levels in an individual from simultaneously measured fasting plasma glucose and fasting plasma insulin levels. This method is based on the assumption that the glucose-insulin axis is central for the metabolic activities, which led to type 2 diabetes. However, significant downregulation of both the *NKX2-1* gene and the *TPD52L3* gene force an increase in the release of free fatty acids (FFAs) into the blood circulation, which leads to a marked reduction in membrane flexibility. These data favor a FFA-glucose-insulin axis. The authors are invited to extend their study with the introduction of the saturation index (number of carbon-carbon double bonds per 100 fatty-acyl chains), as observed in erythrocytes.

Key Words: Free fatty acids; Membrane flexibility; *NKX2-1* gene; RNA sequencing; Type 2 diabetes; *TPD52L-3* gene; Unsaturation index

Core Tip: A substantial reduction in both NKX2-1 and TPD52L3 proteins is largely responsible for a reduction in carbon-carbon double bonds of phospholipids which, in turn, translates into the redistribution of the lateral pressure profile, and thereby reduces the transport speed of glucose molecules across the cell membrane. Consequently, the amount of plasma glucose entering the β-cell *via* GLUT2 gives a false negative result. Also the redistribution of the lateral pressure profile lowers the insulin release from β-cells into the blood circulation. Both phenomena cause the onset of type 2 diabetes mellitus.

Citation: Weijers RN. Free fatty acids, glucose, and insulin in type 2 diabetes mellitus. *World J Diabetes* 2022; 13(3): 275-277
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/275.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.275

TO THE EDITOR

In the August 2021 issue of *World J Diabetes*, Xu *et al*[1] reported on the association of β-cell function and insulin resistance with pediatric type 2 diabetes among Chinese children. The term "insulin resistance" in the article needs additional clarification and review.

As early as 1933, there was as yet no general agreement as to the definition of the term "insulin resistance" and thus gaps in research and clinical care persisted. The breakthrough of the correct description of the term was a clear example of serendipity. A study by Takematsu *et al*[2] compared genome-wide changes in the gene expression in skin between patients with type 2 diabetes and non-diabetic patients *via* RNA sequencing, resulting in the identification of 64 significantly upregulated genes and 120 significantly downregulated genes. Among these regulated genes, the most downregulated gene was *NKX2-1*, with a down regulation value of 3.7×10^{-9}, and in the metabolism category the most downregulated gene was *TPD52L3*, also with a down regulation value of 3.7×10^{-9}. The latter gene has not been linked to type 2 diabetes.

Defective NKX2-1 production is associated with an essential reduction in the activity of the mitochondrial respiratory chain complex, which reduces ATP production. This idea is supported by the data from a study suggesting that a dysregulation of intramyocellular fatty acid metabolism in the offspring of patients with type 2 diabetes was associated with an inherited defect in mitochondrial oxidative phosphorylation[3]. To restore ATP production, the β-oxidation of fatty acids provides assistance by increasing the levels of plasma free fatty acids (FFAs) *via* hydrolysis. Calculation of the saturation indices (number of cis carbon-carbon double bonds per 100 fatty acyl-chains[4]) of FFAs released from human white fat cells and human plasma FFAs in healthy controls reveals that the index of the former is substantially lower (85.5 and 191.9, respectively; $\Delta = 55.4\%$)[5]. Thus, we can conclude that an increase in the release of FFAs into the blood circulation due to an essential reduction in the activity of the mitochondrial respiratory chain complex leads to a marked reduction in the unsaturated index.

In a previous study, the author found that, in brown adipose tissue, the mitochondrial population exists as two subclasses: cytoplasmic mitochondria that do not adhere to lipid droplets and mitochondria that do adhere to lipid droplets. The lipid droplets are cytosolic storage organelles consisting mostly of neutral lipids and enclosed by a phospholipid monolayer membrane[6]. This monolayer has persistent surface packing defects, whereby neutral lipids are accessible to the aqueous cytoplasm and the blood circulation. The idea is that TPD52L3 covers these defects in healthy individuals. Thus, it seems likely that the significant downregulation of *TPD52L3* causes an increase in FFAs in the blood circulation and also lowers the saturation index.

Thus, we can conclude that the downregulation of *NKX2-1* and *TPD52L3* forces an increase in the release of FFAs into the blood circulation due to the leaky lipid droplets and the essential reduction in mitochondrial oxidative and phosphorylation activity, and thereby reduces the unsaturation index, as demonstrated in impaired glucose tolerance[7] (Table 5 in Weijers[7]), gestational diabetes mellitus[7] (Table 5 in Weijers[7]), and type 2 diabetes[4] (Table 2 in Weijers[4]). These phenomena lead to a marked shift from unsaturated to saturated acyl chains in the membrane phospholipids, which redistributes the lateral pressure profile of the cell membrane[8]. The redistribution of this profile narrows the pore diameter of the transmembrane glucose transport channels of all class I glucose transporter proteins, and thus reduces the rate of transport of glucose molecules across the cell membrane, initiating the onset of type 2 diabetes[7].

The following conclusions can be drawn from the presented information. First, type 2 diabetes is characterized by reduced membrane flexibility in the pancreatic β-cell, which adversely affects the amount of glucose entering the β-cell *via* GLUT2, and thereby lowers the synthesis of the necessary amount of circulating insulin molecules. Secondly, fusion of the insulin-containing granule with the β-cell plasma membrane, followed by the formation of a suitable pore diameter for insulin transport into the blood circulation, requires high flexibility in both the granule-cell membrane and the β-cell membrane. The reduction in the flexibility of both membranes lowers the insulin release from the β-cell into the blood circulation. These facts underline the fact that a reduction in membrane flexibility lowers not only the rate of transport of glucose molecules into the β-cell but also the rate of transport of insulin molecules from the β-cell into the blood circulation.

Up to now, it has been thought that the glucose-insulin axis is central to the metabolic activities that lead to type 2 diabetes. A publication in 1992 is an exception in this respect, having the title: "What if Minkowski had been ageusic? An alternative angle on diabetes"[9]. This study suggested that the basic pathophysiological mechanisms of type 2 diabetes might be more readily understood if viewed in the context of underlying abnormalities of lipid metabolism. Xu *et al*[1] used the HOMA2 model to estimate β-cell function and insulin resistance levels in a pediatric individual from simultaneously measured fasting serum glucose and fasting serum insulin concentrations. The summarized phenomena, in my opinion, are a scientific basis for the idea that membrane flexibility plays an important part in the onset of type 2 diabetes.

Therefore, I suggest that Xu *et al*[1] add to their article a follow-up study including the unsaturation index, as a parameter for membrane flexibility, based on the erythrocyte membrane fatty-acid compositions because, at the most basic level, the basal metabolic rate of a cell is directly linked to its cell

membrane's acyl composition[10]. A strong argument in favor of the FFA-glucose-insulin axis is the observation that in persons at high risk for type 2 diabetes, the incidence of diabetes was reduced by 58% with lifestyle intervention and by 31% with metformin, as compared with placebo[11]. It seems likely that physical activity, after all, raises the levels of the unsaturation index, in contrast to metformin.

FOOTNOTES

Author contributions: Weijers RNM solely contributed to this paper.

Conflict-of-interest statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Country/Territory of origin: Netherlands

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S-Editor: Zhang H
L-Editor: Webster JR
P-Editor: Zhang H

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World Journal of Diabetes

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World J Diabetes 2022 March 15; 13(3): 278-281

DOI: 10.4239/wjd.v13.i3.278

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Beyond diabetes remission a step further: Post bariatric surgery hypoglycemia

Devraj Lath, Kripa Elizabeth Cherian, Thomas Vizhalil Paul, Nitin Kapoor

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Belosludtseva NV, Gupta R, Liu C, Seetharaman RV, Shao JQ

Received: September 24, 2021
Peer-review started: September 24, 2021
First decision: January 12, 2022
Revised: January 21, 2022
Accepted: February 23, 2022
Article in press: February 23, 2022
Published online: March 15, 2022

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Abstract

Postbariatric hypoglycemia is a rare but increasingly recognized complication of bariatric surgery, with significant associated morbidity, and many patients often require multimodal treatment. A mixed meal challenge test is often helpful to diagnose this condition. This manuscript highlights the underlying mechanisms that lead to this condition and the novel emerging therapeutic targets that target these mechanisms.

Key Words: Postbariatric hypoglycemia; Hyperinsulinemic hypoglycemia; Avexitide; GLP-1 antagonist; Obesity

Core Tip: Postbariatric hypoglycemia is an uncommon complication presenting months to years after bariatric surgery (mostly in Roux-en-Y gastric bypasses) as postprandial hyperinsulinemic hypoglycaemia occurring 1-3 h after meals, and the associated neuroglycopenic symptoms can be incapacitating. Medical nutrition therapy forms the foundation of management, with pharmacotherapy and surgical interventions available for those who do not respond. An increased understanding of the implicated mechanisms has led to the development of targeted agents like avexitide, which has demonstrated good efficacy in a Phase 2 clinical trial (PREVENT) recently.

Citation: Lath D, Cherian KE, Paul TV, Kapoor N. Beyond diabetes remission a step further: Post bariatric surgery hypoglycemia. *World J Diabetes* 2022; 13(3): 278-281
URL: https://www.wjgnet.com/1948-9358/full/v13/i3/278.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i3.278

TO THE EDITOR

We read with interest the review by Jin *et al*[1], who discussed the potential mechanisms underlying the remarkable efficacy of bariatric surgery in inducing remission of type 2 diabetes mellitus, ranging from 33% in adjustable gastric banding to up to 95% in biliopancreatic diversion. In a recent meta-analysis involving 174772 patients compared in 16 cohort studies and 1 controlled trial, bariatric surgery was associated with a reduction in the risk of all-cause mortality by 49.2% and an increased median life expectancy of 6.1 years. These benefits were even greater among those diagnosed with type 2 diabetes mellitus[2].

Postbariatric hypoglycaemia (PBH) is an infrequent but potentially debilitating complication, with a multicenter registry-based study in Spain having reported 22 patients developing hypoglycemia following 4645 interventions, amounting to an incidence rate of 0.47%[3]. In another study using registry data, 5040 Swedish patients that underwent Roux-en-Y gastric bypass (RYGB) were matched with 10 non-surgical controls each, with no preoperative difference in the frequency of hypoglycemia or potentially related diagnoses such as confusion, seizures or syncope[4]. Following gastric bypass, 0.2% of the post-gastric bypass cohort were admitted for hypoglycemia *vs* 0.04% of the general population. Although the overall incidence is variable, these patients were at a two- to sevenfold increased risk of hypoglycemia and related diagnoses when compared to their controls. The authors also found that there was no significant increase in the risk of postbariatric hypoglycemia or related diagnoses among patients undergoing restrictive procedures, namely vertical banded gastroplasty (4366) and gastric banding (2917) when matched with controls. Patients without diabetes especially are at an increased risk of hypoglycemia following bariatric surgery *vs* those managed medically[5]. Greater frequencies of hypoglycemia (32.6% and 22.6%) are observed in gastric bypass (GBP) and sleeve gastrectomy patients subjected to a two-hour oral glucose tolerance test (OGTT)[6], with much lower rates (2.3%)[6] reported in those undergoing gastric banding[5-6]. In another study by Tzovaras *et al*[7], 29% experienced definite dumping syndrome while another 16% had symptoms suggestive of the same.

Patients undergoing bariatric surgery (especially RYGB surgery) may develop severe vasomotor symptoms of sweating, dizziness, weakness and flushing, referred to as dumping syndrome. These are attributed to the osmotic effect of rapid food entry into the intestines, release of peptide hormones like vasoactive intestinal peptide, incretins and the enteric neural response. By contrast, the development of symptoms such as confusion, decreased vision, syncope, hunger, behavioural changes, syncope and seizures are suggestive of neuroglycopenia, and these patients are found to have low plasma glucose levels 1-3 h after a meal consistent with reactive hypoglycemia[8]. This occurs months to years after bariatric surgery, and though these phenomena have been classified as early and late dumping syndrome respectively[8], some suggest the term postbariatic hypoglycemia be used instead[8-9] and that the term dumping syndrome be reserved for the vasomotor symptoms caused by rapid gastric emptying, diagnosed by an increase in pulse rate > 10/min and/or a rise in hematocrit by 3% after an OGTT. Apart from the risks of severe hypoglycemia, these patients are also more likely to regain weight due to frequent food intake.

The diagnosis of hypoglycaemia requires the documentation of low plasma glucose during the presence of symptoms and/or signs attributable to hypoglycemia, which are relieved by raising the plasma glucose concentration (known as Whipple's triad)[10]. Postbariatric hypoglycemia following meal intake is caused by postprandial hyperinsulinemia, diagnosed by a mixed meal challenge test (MMCT) demonstrating hypoglycemia (glucose less than 55 mg/dL) accompanied by inappropriately elevated insulin (> 3.0 U/mL) and C-peptide (> 0.6 ng/mL)[9-10]. An important differential is the exclusion of a co-existing insulinoma[11-12] by cross sectional imaging or endoscopic ultrasonography, although these patients generally present with fasting hypoglycemia.

Postprandial hyperinsulinemic hypoglycemia following bariatric surgery was first described by Service *et al*[11] in a series of six patients who presented years after GBP surgery with neuroglycopenic symptoms and were found to have hyperinsulinemic hypoglycaemia. One patient was found to have an insulinoma, and the other five underwent pancreatectomy guided by intra-arterial calcium stimulation tests. Pathological examination showed islet cell hypertrophy and hyperplasia suggestive of nesidioblastosis and it was initially proposed that bypass surgery had resulted in beta cell hyperfunctioning and hyperinsulinemia. However other studies contest this finding[13], and other mechanisms proposed include an enhanced incretin effect[14], abnormal counter-regulatory hormone responses[15], altered enterohepatic circulation of bile acids[16] and changes in the microbiome[17]. The rapid transit of food from the stomach to the intestinal L cells is believed to result in an excessive release of incretins such as gastric inhibitory peptide and glucagon-like peptide 1 (GLP-1) in particular, with greater levels being

observed in symptomatic patients after meals[14].

The management of PBH is complex as its mechanisms remain incompletely understood. The majority of cases exhibiting mild symptoms respond to dietary modification, and medical nutritional therapy (MNT) is the cornerstone of management. The frequent intake of smaller meals comprising carbohydrates with a low glycaemic index helps prevent hypoglycaemia[18], with the intake of meals low in protein and/or high in sugars known to trigger these episodes[19]. Various pharmacological agents have been used with some success for patients who fail MNT, by blunting the inappropriately elevated insulin secretion and ensuing hypoglycemia. These include the alpha-glucosidase inhibitor acarbose, calcium channel antagonists like nifedipine or verapamil, the beta-cell adenosine triphosphate-sensitive potassium channel agonist diazoxide (inhibits insulin secretion by hyperpolarisation) and somatostatin analogues like octreotide[20]. Refractory patients may require a gastrostomy tube placement or a restrictive procedure, with some undergoing partial or total reversal of the bypass [9,20]. Over the years, GLP-1 has become an increasingly attractive target. A recent phase 2 randomised placebo-controlled crossover study (PREVENT) employing the GLP-1 receptor antagonist avexitide [exendin (9-39)] for 28 days showed a significant decrease in the occurrence of hypoglycemia in response to a MMCT requiring rescue as well as on continuous glucose monitoring, with an improved glycemic profile[21]. In another study[22], 12 participants with PBH were randomised to receive either glucagon or a placebo from an artificial pancreas system during meals as guided by a predictive algorithm using continuous glucose monitoring. The patients who received glucagon did not require rescue glucose or develop severe hypoglycemia (< 55 mg/dL), unlike those who received the vehicle, and thus mitigating severe hypoglycemia in PBH.

Elucidation of the other proposed mechanisms may guide the development of other safe and effective therapies for PBH.

FOOTNOTES

Author contributions: Kapoor N conceived of the presented idea and provided critical feedback to the final manuscript; Lath D wrote the manuscript; Cherian K and Paul TV approved the main conceptual ideas and proof outline; all authors provided final edits and approved the manuscript.

Conflict-of-interest statement: No conflict of interest.

Country/Territory of origin: Australia

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S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com

ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 April 15; 13(4): 282-386

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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World Journal of Diabetes

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The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

PUBLICATION DATE
April 15, 2022

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 282-307

DOI: 10.4239/wjd.v13.i4.282

ISSN 1948-9358 (online)

REVIEW

Insulin-resistance in paediatric age: Its magnitude and implications

Mohammed Al-Beltagi, Adel Salah Bediwy, Nermin Kamal Saeed

Specialty type: Pediatrics

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A, A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Herold Z, Hungary; Liao JX, China; Wang CR, Taiwan

Received: February 18, 2022
Peer-review started: February 18, 2022
First decision: March 11, 2022
Revised: March 12, 2022
Accepted: March 27, 2022
Article in press: March 27, 2022
Published online: April 15, 2022

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Abstract

Insulin resistance (IR) is insulin failure in normal plasma levels to adequately stimulate glucose uptake by the peripheral tissues. IR is becoming more common in children and adolescents than before. There is a strong association between obesity in children and adolescents, IR, and the metabolic syndrome components. IR shows marked variation among different races, crucial to understanding the possible cardiovascular risk, specifically in high-risk races or ethnic groups. Genetic causes of IR include insulin receptor mutations, mutations that stimulate autoantibody production against insulin receptors, or mutations that induce the formation of abnormal glucose transporter 4 molecules or plasma cell membrane glycoprotein-1 molecules; all induce abnormal energy pathways and end with the development of IR. The parallel increase of IR syndrome with the dramatic increase in the rate of obesity among children in the last few decades indicates the importance of environmental factors in increasing the rate of IR. Most patients with IR do not develop diabetes mellitus (DM) type-II. However, IR is a crucial risk factor to develop DM type-II in children. Diagnostic standards for IR in children are not yet established due to various causes. Direct measures of insulin sensitivity include the hyperinsulinemia euglycemic glucose clamp and the insulin-suppression test. Minimal model analysis of frequently sampled

intravenous glucose tolerance test and oral glucose tolerance test provide an indirect estimate of metabolic insulin sensitivity/resistance. The main aim of the treatment of IR in children is to prevent the progression of compensated IR to decompensated IR, enhance insulin sensitivity, and treat possible complications. There are three main lines for treatment: Lifestyle and behavior modification, pharmacotherapy, and surgery. This review will discuss the magnitude, implications, diagnosis, and treatment of IR in children.

Key Words: Insulin resistance; Children; Diabetes mellitus; Obesity; Genetic; Acquired

Core Tip: Insulin resistance (IR) increases in children due to lifestyle changes and the pandemic of obesity. There is a strong association between obesity in children and adolescents and IR. There is a broad range of genetic and acquired causes of IR with a wide variability of its prevalence from one country to another. Many available tests can directly or indirectly estimate IR. To prevent future IR, we should target all the factors that could help the development of IR, especially obesity.

Citation: Al-Beltagi M, Bediwy AS, Saeed NK. Insulin-resistance in paediatric age: Its magnitude and implications. *World J Diabetes* 2022; 13(4): 282-307
URL: https://www.wjgnet.com/1948-9358/full/v13/i4/282.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i4.282

INTRODUCTION

Initial data suggest that insulin resistance (IR) is becoming more common in children and adolescents than before. IR is insulin failure in the normal plasma levels to adequately stimulate glucose uptake by the peripheral tissues such as adipose tissues and skeletal muscle, inhibit the hepatic gluconeogenesis and release of glucose into circulation, and/or suppress the output of very-low-density lipoprotein[1]. IR is a spectrum disorder that ranges from very mild to very high resistance and is commonly associated with obesity. Consequently, chronic hyperinsulinemia occurs as a compensatory mechanism to IR[2]. There is a strong association between obesity in children and adolescents, IR, and the metabolic syndrome (MS) components, including the high risk of cardiovascular complications[3]. IR syndrome (IRS) is characterized by the presence of hyperinsulinemia and one or more of the following: impaired glucose tolerance, central obesity, hypertension, hirsutism, hypertriglyceridemia, hypercholesterolemia, reduced high-density lipoprotein, high low-density lipoprotein, hyperuricemia, coagulation abnormalities favoring thrombosis, polycystic ovary syndrome and/or menstrual disturbances[4]. Table 1 shows various forms of IR.

EPIDEMIOLOGY OF IR IN CHILDREN

IR is rising due to increasing obesity among children and adolescents, changing the lifestyle with lack of physical activities, high-calories intake (western diet style), overdependence on the technology with more sedentary life due to TV watching, and social media addiction. Although IR usually occurs in obese people, not all obese people have IR. It may also occur in normal physiological status during puberty or pregnancy[5]. IR may appear as early as two years of age in children with certain genetic predisposition and environmental influences (*e.g.*, decreased activity) with a peak at puberty due to increased growth hormone secretion[6]. IR prevalence in children varies from one country to another depending on many factors, including genetic, racial, and environmental factors, and due to the heterogeneity of the methods of data collection and the cut-off values used to define IR. In a systematic review study by van der Aa *et al*[7], the overall prevalence rates of IR ranged between 3.1% and 44% of children and adolescents, being more prevalent in girls than boys due to their earlier pubertal changes. They also found that IR reached 68.4% in obese boys. Jurkovičová *et al*[8] show an IR prevalence rate of 18.6% in Slovakian adolescents with significant association with insufficient physical activity, low level of physical fitness, a small number of daily meals and breakfast skipping, more sweetened beverages consumption, and low educational level of fathers.

IR is higher in urban than rural children and among Hispanics, American Indians, African Americans, East Asians, and South Asians than white European adolescents. However, it is increasingly observed across all racial boundaries, particularly with the increasing obesity rate. In a cross-sectional

Table 1 Various types of insulin resistance in children

IR type	Description
Partial IR	The impairment of insulin receptor expression is limited to specific tissue and consequently exhibits some features of insulin resistance according to the tissue affected
Complete IR	The impairment of insulin receptor expression is extensively distributed all over the body tissues and organs with the full expression of the syndrome
IR syndrome type A	It is a rare type of hereditary insulin resistance syndrome due to the lack of response of the tissues to the insulin. Patients with this syndrome are nonobese and demonstrate severe hyperinsulinemia, hyperandrogenism, and acanthosis nigricans. The clinical features are more severe in affected females than in males, and they mostly become apparent at the age of puberty
IR syndrome type B	It is a rare disorder caused by autoantibodies to the insulin receptor. This disorder is most frequently reported in middle-aged black women and is invariably associated with other autoimmune diseases
Compensated IR	The resulting hyperinsulinemia compensates for the body's metabolic needs and prevents metabolic derangement
Non-compensated IR	There is a progressive failure of compensatory hyperinsulinemia to fulfill the body's metabolic needs through puberty with rising blood glucose and triglyceride levels and metabolic derangement
Early childhood IR	Onset before the age of ten, a metabolic syndrome diagnosis cannot be made, but further measures should be taken if one of the parents has metabolic syndrome, DM type-II, dyslipidemia, cardiovascular risk factors, hypertension, or obesity
Late childhood IR	Onset after ten years of age, diagnosis of metabolic syndrome can be made
Social IR	It is a negative attitude directed towards avoiding or rejecting insulin therapy by some social groups

DM: Diabetes mellitus; IR: Insulin resistance.

study of urban Indian schoolchildren, Das *et al*[9] found that the overweight or obesity rates were 28.2%; about 21.8% of them had IR. Adolescence increases the risk of IR. Arslanian *et al*[10] showed that the IR rate was higher in obese adolescents than in obese adults, despite similar degrees of adiposity and glycaemic status. This observation could explain the relatively poor response of these obese children to metformin and the rapid decline of β-cell function observed in adolescents than in adults with diabetes mellitus (DM) type-II. IR shows marked variation among different races. Raygor *et al*[11] show that the overall IR was less in non-Hispanic Whites and African Americans than East Asians and South Asians. Ehtisham *et al*[12] also showed that South Asian adolescents have significantly more IR and body fat than white European adolescents, which may increase their risk of developing DM type-II. They attributed these racial differences to the ethnic differences in the composition of children's body fat. These racial differences are crucial to understanding the possible cardiovascular risk, specifically in high-risk races or ethnic groups.

PATHOGENESIS OF IR

IR is multifactorial.

PHYSIOLOGIC EFFECTS OF INSULIN

Insulin is a hormone produced from proinsulin in the beta cells of the pancreas when stimulated by elevated blood glucose. Proinsulin is broken apart, leaving insulin and C-peptide. Both are secreted and enter the bloodstream in equimolar amounts. Because insulin and C-peptide are equally secreted, both can be used to quantify endogenous insulin production. Average fasting serum C-peptide or insulin values are around 0-30 μIU/mL. The average daily insulin requirement is 0.5-0.7 units/kg of body weight[3]. Insulin stimulates the amino acids' entry into body cells. It enhances protein synthesis and fat storage and prevents fat mobilization for energy. It also promotes glucose entry into cells as an energy source. It has euglycemic effects by promoting glucose storage as glycogen in muscle and liver cells and inhibiting glucose production from liver or muscle glycogen from non-carbohydrates[13]. Insulin action starts by binding to a surface glycoprotein receptor expressed on the target cell's surface. This insulin receptor is composed of an alpha-subunit and a beta-subunit. Alpha unit binds the insulin, while beta-subunit is a tyrosine-specific protein kinase stimulated upon binding insulin to alpha subunit. This kinase activation generates a specific signal that ultimately results in insulin's effects on glucose, protein, and lipid metabolism. Insulin also mediates its growth-promoting effects by activating receptors linked to insulin-like growth factors[14]. IR does not necessarily involve all the insulin-dependent pathways

(partial IR). In partial IR, some manifestations such as hyperinsulinemia, hyperglycemia, hyperandrogenism, ovulatory dysfunction, soft tissue overgrowth, and acanthosis nigricans may present while the patients may have average lipid profiles[15,16].

GENETIC BASIS FOR IR

As many molecular pathways are concerned with energy homeostasis, protein and lipid metabolism, and insulin receptor functioning mechanism, many genetic mutations can end with IR development (Table 2). Insulin receptor mutations, mutations that stimulate autoantibody production against insulin receptors, or mutations that induce the formation of abnormal glucose transporter 4 (GLUT4) molecules or plasma cell membrane glycoprotein-1 molecules; all cause abnormal energy pathways and end with the development of IR. Mutations in the lipid pathway such as mutations in the adipocyte-derived hormones or their receptors (leptin, adiponectin, resistin), mutations in the peroxisomal proliferator-activated receptors (α, γ, δ), the mutation in the lipoprotein lipase gene, and other genes concerned with adipose tissue formation; all these mutations have a significant role in the development of IR. At the same time, mutations in proteases and serpin protease inhibitors cause IR and DM type II. The *CAPN10* gene is also engaged in GLUT4 vesicle translocation during the insulin-stimulated glucose uptake by adipocytes; it is also associated with IR and type 2 diabetes[17]. These mutations could occur in heterozygous or homozygous forms. The occurrence of several heterozygous mutations in the same person (a compound heterozygote) even when recessive; could have additive effects and produce significant consequences[18,19]. Insulin receptor pathway defects may occur due to mutations of the insulin receptor gene, causing a broad spectrum of inherited IRS, including type A syndrome of extreme IR, leprechaunism, Rabson-Mendenhall syndrome, and polymorphism in plasma cell membrane glycoprotein-1[20]. Insulin-like growth factor 1 (somatomedin C or IGF-1) is a hormone produced mainly by the liver, like insulin in the molecular structure, and plays a crucial role in childhood growth.

Growth hormone (GH) stimulates IGF-1 production[21]. Low IGF-1 levels are associated with many conventional cardiovascular risk factors related to increase IR. Kuang *et al*[22] found that obese prepubertal boys had lower IGF-1 standard deviation scores than boys without obesity and that whole-body insulin sensitivity index was positively correlated with IGF-1. Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated transcription factors of the nuclear hormone receptor superfamily comprising of the following three subtypes: PPARα, PPARγ, and PPARβ/δ. Activation of PPAR-α reduces triglyceride levels and is involved in regulating energy homeostasis. Activation of PPAR-γ causes insulin sensitization and enhances glucose metabolism, whereas activation of PPAR-β/δ enhances fatty acids metabolism. Thus, the PPAR family of nuclear receptors plays a significant regulatory role in energy homeostasis and metabolic function. Mutations of this family induce IR[23,24].

ACQUIRED CAUSES OF IR

The parallel increase of IRS with the dramatic increase in the rate of obesity among children in the last few decades indicates the importance of environmental factors in increasing the rate of IR. Acquired causes of IR include lack of physical activity, exogenous obesity due to excess food intake, drugs, glucose toxicity due to hyperglycemia, increased free fatty acids, and the aging process. Puberty itself is occasionally associated with IR[25]. The development of polyclonal autoantibodies against insulin receptors preventing insulin from its action is a rare condition known as type B IRS, which should be distinguished from type A IRS[26]. IR may occur due to excess insulin antagonists in excessive steroid production such as Cushing syndrome, acromegaly, and stressful situations such as severe infection, trauma, surgery, uremia, diabetes ketoacidosis, and liver cirrhosis. Certain medications may also increase the risk of IR, such as glucocorticoid therapy, niacin, cyclosporine, and protease inhibitors[27]. Treatment with growth hormone can elicit transient IR. High sodium consumption causes hypertension, enhanced glucocorticoid production, and IR[28]. Protease inhibitor used as a part of anti-human immunodeficiency virus therapy is associated with lipodystrophy and IR. Nucleoside analogs, *e.g.*, acyclovir and abacavir, may also induce IR[29]. Insulin therapy can induce anti-insulin antibody formation, which is usually present in low titers in most patients. However, in rare cases, these antibodies can cause significant IR (pre-receptor or insulin-autoimmune syndrome) with enhanced insulin destruction at the subcutaneous injection site[30].

RISK FACTORS FOR IR

Alongside the genetic factors that play a fundamental role in the development of IR, other factors could have significant contributing effects. Babies born for mothers with DM, whether pregestational or gestational, are at risk for future development of impaired insulin sensitivity and obesity even when

Table 2 Various causes of the genetic type of insulin resistance

Site of defect	Type of defect	Clinical features
Insulin receptor: Mutations in the *INSR* gene (19p13.2) → faulty insulin receptor that cannot transmit signals properly	Type A IR syndrome mutation in the *INSR* gene (19p13.2)	Females are more affected than males. Appear during adolescence (delayed menses, 1ry amenorrhea, oligomenorrhea, hirsutism, acanthosis nigricans). Some males may have hypoglycemia & occasionally acanthosis nigricans. Later, they may develop DM
	Leprechaunism or Donohue syndrome (extremely rare) autosomal recessive	Extreme insulin resistance with fasting hypoglycemia and postprandial hyperglycemia, low birth weight, distinctive craniofacial features (bulging eyes, protuberant and low-set ears, thick lips, and upturned nostrils), skin abnormalities (hyperkeratosis), enlargement of the breast and clitoris in females and the penis in males, growth delays, & features of other endocrinopathies
	Rabson-Mendenhall syndrome (rare) autosomal recessive, also include Donohue syndrome	Severe insulin resistance, low birth weight, failure to thrive, lack of subcutaneous fat, muscle wasting, dental abnormalities; hirsutism, polycystic ovaries in females; enlargement of the nipples, genitalia, kidneys, heart, and other organs. Most affected individuals also have acanthosis nigricans, and distinctive facial features include prominent hypertelorism; a broad nose; and large, low-set ears
	Polymorphism in PC-1	It causes PC-1 overexpression to reduce autophosphorylation of the insulin receptor β-subunit, impairs insulin stimulation of insulin receptor activation & downstream signaling with short at birth, hyperinsulinemia, and high insulin resistance, high prevalence of diabetes, hypertension, and preeclampsia
Defects in fat cell and lipid homeostasis pathway	Congenital generalized lipodystrophy (mutations in *BSCL2* on 11q13, & *AGPAT2* gene on 9q34)	Autosomal recessive, extreme lack of body fat, and severe insulin resistance since birth
	Kobberling's syndrome (mutation in the *PPAR-δ* gene) FPL type 1	X-linked dominant, lethal in the hemizygous male. The autosomal dominant form of familial partial lipodystrophy was also described, characterized by the absence of subcutaneous fat, and presence of adipose tissue inside the body cavities and skeletal muscle hypertrophy. Fat loss is generally confined to the arms and legs. Fat loss is usually more prominent on the arms and legs' lower (distal) portions than proximal
	Dunnigan's syndrome (*LMNA* gene mutation) (1q22) FPL type 2	An X-linked dominant, lethal in hemizygous males, present with partial lipodystrophy characterized by sparing of the face. The onset of lipodystrophy usually occurs at or around puberty, with improper fat distribution (loss of fat in the limbs and gluteal region and variable regional fat accumulation on the face, neck, and axillary regions giving patients a cushingoid appearance). Females often have a more severe phenotype than males. An increased skeletal muscle volume and mass are also noted. Prominent veins (due to lipoatrophy) are noted in the limbs
	Allelic variation in PPAR-δ, PPAR-α, polymorphism of *UCP1*, *UCP2*, *UCP3* genes & polymorphism of β-2 and β-3 adrenergic receptor	Allelic variation in PPAR-δ influences body fat mass by effects on adipocyte; polymorphisms of *PPAR-α* gene can lead to higher triglyceride and insulin levels; polymorphism of the lipoprotein lipase gene was both linked and associated with insulin resistance; polymorphism of *UCP1*, *UCP2*, *UCP3* genes are associated with marked adiposity and DM type II; and polymorphism of β-2 and β-3 adrenergic receptors associated with chronic non-communicable disorders, such as cardiovascular diseases, asthma, chronic obstructive pulmonary disease, and obesity, as well as β-agonists and antagonists response and toxicity
Proteases CALP10	*CALP10* gene polymorphism	It is associated with reduced muscle mRNA levels and insulin resistance, metabolic syndrome, type II DM, and polycystic ovary syndrome
Other hormonal disorders	*POMC* mutations	Causes mutations in the *POMC* gene were linked with a clinical phenotype of adrenal insufficiency, red hair pigmentation, early-onset and rapidly progressive obesity, early-onset type 2 diabetes, hypothyroidism, hypogonadism, and growth hormone deficiency
	The *MC4R* gene mutations	MC4R mutations are the most common form of monogenic obesity and have been implicated in 1% to 6% of early-onset severe obesity
	The *MC3R* gene mutations	Inactivating mutations in the *MC3R* gene causes obesity in mice but is not clear in human
	Leptin and leptin receptor mutations	Homozygous leptin gene mutations are associated with the early onset of severe obesity and diverse impairment of physiological functions. Recessive leptin receptor mutations are associated with similar pathology in the homozygous state
	Ghrelin polymorphisms	Ghrelin is an orexigenic peptide that stimulates appetite and induces body weight gain and adipogenesis. Ghrelin polymorphisms may be associated with obesity and obesity-related phenotypes
	NPY gene	*NPY* gene polymorphism is associated with an increased risk of metabolic syndrome and its related phenotypes, such as central obesity and hyperglycemia
	CART polymorphisms	*CART* gene polymorphism is associated with a genetic predisposition to insulin resistance & obesity
	ER mutations	*ER* mutations cause impaired insulin sensitivity/glucose intolerance, hyperinsulinemia, and obesity
Prader-Willi syndrome	15q11.2–q12, uniparental maternal disomy	A key feature of Prader-Willi syndrome is a constant sense of hunger (hyperphagia) that usually begins at about 2 years of age with several physical, mental, and behavioral problems

Alström syndrome	Mutations in the *ALMS1* gene	The *ALMS1* gene provides instructions for making a protein whose function is unknown. *ALMS1* gene mutants in the hypothalamus might lead to hyperphagia followed by obesity and insulin resistance
Bardet-Biedl syndrome	Mutations in at least 14 different genes (often called *BBS* genes)	Vision loss is one of the significant features of Bardet-Biedl syndrome. Obesity is another characteristic feature of Bardet-Biedl syndrome. Abnormal weight gain typically begins in early childhood and continues to be an issue throughout life
Cohen syndrome	Mutations in the *VPS13B* gene (also called the *COH1* gene)	Cohen syndrome is an inherited disorder that affects many parts of the body and is characterized by developmental delay, intellectual disability, microcephaly, and weak muscle tone (hypotonia). Obesity develops in late childhood or adolescence
Biemond syndrome II		Biemond syndrome type II is a rare genetic neurological & developmental disorder reported in few patients with a poorly defined phenotype, including iris coloboma, short stature, obesity, hypogonadism, and postaxial polydactyly, and intellectual disability

DM: Diabetes mellitus; IR: Insulin resistance; PPAR: Peroxisome proliferator-activated receptor. UCP1, UCP2 and UCP3 are the uncoupling protein homologs.

they have an average birth weight[31]. The presence of hyperglycemia in pregnant mothers even without other signs of gestational diabetes is a mere risk factor for future IR and obesity in the offspring [32]. Children born to mothers with DM type I are more prone to have DM type II than children born with paternal DM type I[33]. Although many babies of mothers with gestational diabetes have excess body fat, the association of excess adiposity observed in these babies with the future development of IR is controversial[31]. The large birth size shows no association with later development of IR and impaired β-cell function in infancy. However, Huang *et al*[34] showed that the growth pattern during infancy could be related to the development of IR as decelerated infancy growth may be unfavorable to beta-cell function.

IR risk is high in newborns with small gestational age (SGA). However, studies showed that IR was not related to the birth weight but was related to the rate of weight gain during catch-up growth, especially in girls[34]. SGA may also result from IR's genetic causes as insulin is a potent antenatal growth hormone. Children born as SGA tend to have more intra-abdominal visceral fat than those with appropriate weight for age, even before the development of obesity. They are at more risk of having IRS in adolescence[35,36].

Consequently, SGA could be one of the manifestations of inherited IR with diminished fetal growth [37]. So, the relation between the birth weight and the risk of IR may follow a U-shaped relation[38]. Dabelea *et al*[39] showed that Pimas with low birthweight are thinner by 5-29 years. However, they are more insulin resistant and more liable to have DM type II than Pimas with average birth weight.

On the other hand, Pimas with high birth weight are more liable to be obese but less liable for IR regarding their body size[39]. Murtaugh *et al*[40] also observed similar findings. They showed a U-shaped relation between birth weight, body mass index (BMI), and fat mass in adolescents. So, children who rapidly gain weight are more liable to have IR, including preterm babies, during their rapid catch-up growth[41]. Ethnicity could pose a significant risk for IR as ethnicity and race affect glucose metabolism and insulin regulation. However, there are no international guidelines to address these racial/ethnic effects and recommend specific clinical advice[42]. Puberty is a physiological risk factor for IR due to various metabolic and hormonal changes. Insulin sensitivity drops by 25%-50% during puberty, reaching nadir by mid-puberty, then normalizes by the end of puberty. However, occasionally, puberty-induced IR does not resolve by the end of puberty, especially in adolescents who are obese, increasing the cardiometabolic risk of IR[43].

Deficiency of vitamin D is associated with many chronic conditions and diseases, including obesity, and increased metabolic dysregulation severity, such as IR and hyperlipidemia. Vitamin D performs a crucial role in the adipogenesis process and inflammatory condition in adipocytes and adipose tissue. Additionally, vitamin D can regulate adipocyte apoptosis and the gene expression responsible for the adipogenesis process, oxidative stress, inflammation, and metabolism in mature adipocytes. An adequate 1,25-dihydroxyvitamin D3 level is essential for normal insulin secretion[44,45]. Pires *et al*[46] showed that vitamin D deficiency in children with overweight or obesity increases the risk of IR during puberty.

Obesity is the most predominant pathophysiological risk factor of IR. IR positively correlated with the body mass index and proportion of body fat. Children with overweight or obesity have lower insulin sensitivity than children with average body weight[47]. The body fat distribution is also a significant risk factor that can predict IR. Although subcutaneous and visceral adipose tissues are related to IR, visceral adipose tissues more strongly correlate with IR than subcutaneous adipose[48]. Both subcutaneous and visceral adipose tissues secrete free fatty acids into the blood, correlated with the fatty mass. The higher the plasma-free fatty acid levels are, the more will be the IR. Visceral adipose tissues have more glucocorticoid receptors and elevated local glucocorticoids concentrations, which contribute to the development of metabolic screen[49]. In addition, visceral adipose tissues correlate with adiponectin levels, the degree of endothelial dysfunction and blood levels of C-reactive protein

(CRP), interleukin-6 (IL-6), and degree of systemic inflammation. In addition, ectopic non-visceral or subcutaneous fat deposition, such as intramyocellular fat in adolescents with obesity, is also associated with reduced peripheral insulin sensitivity[50].

Sex affects the impact of fat distribution on the development of fat resistance. In males, abdominal subcutaneous and visceral adipose tissues are associated with IR, while in females, visceral adipose tissues are associated with IR and insulin secretion. The lifestyles such as physical activity and nutritional behavior; have a poorly defined relationship with insulin sensitivity in the pediatric age[51]. However, increased caloric intake is a leading cause of obesity, IR, and hyperinsulinemia. A saturated fatty diet and sweetened beverages could be associated with altered insulin sensitivity and secretion. However, Weigensberg *et al*[52] showed that these changes were more observed in black but not white children, related to underlying ethnic differences. The consequence of lack of physical activity on IR, independent of weight changes and adiposity, remains debated. Marson *et al*[53] showed that exercise training, especially aerobic training, reduces the fasting insulin levels and IR indices in children and adolescents with obesity or overweight and may prevent the development of the MS and DM type II.

Some diseases make the child more susceptible to more increased risk of IR. Boys with Klinefelter syndrome may have truncal obesity, IR, and other features of MS as early as 4–12 years due to reduced physical activity[54]. In children with asthma and obesity or overweight, there is an increased risk of IR, which, together with obesity and MS, worsens lung function. These factors interact together, making asthma control more difficult[55]. Obstructive sleep apnea (OSA) is a common comorbidity in children with obesity. OSA induces sympathetic activation and enhances the development of IR[56]. In addition, the use of certain medications could increase the risk of IR. Systemic steroids used in managing various disorders commonly have significant adverse effects on body weight and insulin sensitivity[57]. Various psychotropic medications induce significant weight gain and IR, commonly observed soon after therapy [58].

CONSEQUENCES OF IR IN PEDIATRIC AGE

Most patients with IR do not develop DM type-II. However, IR is a crucial risk factor to develop DM Type-II in children. Two critical factors are needed to develop DM type-I: impaired β-cell function and IR[59]. The genetic basis of the patients determines the response of pancreatic β-cells to hyperinsulinism and IR. Children and adolescents with obesity and IR are more liable to impaired glucose tolerance than those with obesity but without IR[60]. However, Cali *et al*[61] showed that children with obesity who developed impaired glucose tolerance had a primary defect in β-cell function and insulin-resistant presence just served as an aggravating factor. The risk to develop DM type II can be predicted using the disposition index as IR alone is not enough to expect the risk of DM type II. This index measures the ability of the beta cell to secrete insulin in response to a glucose load.

Consequently, children with IR and hyperinsulinemia are at increasing cardiometabolic risk of developing MS in the different ethnic groups[62]. IR is associated with high levels of circulating endothelial dysfunction biomarkers (E-selectin and intercellular adhesion molecule) and decreased antiatherogenic adipocytokine adiponectin levels[63]. Children who have IR and hyperinsulinemia and did not develop type II DM are still at risk for other complications of IR such as dyslipidemia, early atherosclerosis, hypertension, progressive obesity (especially centripetal type), fatty liver infiltration, hypercoagulation, skin disorders such as acanthosis nigricans and increased skin tags, Polycystic ovary syndrome, renal impairment in the form of focal segmental glomerulosclerosis, and an increased risk of cancer[64]. Accordingly, IR should not be considered a benign condition even in the absence of DM type-II.

Although obesity (especially centripetal type) could lead to IR, genetically based IR may also provoke the progression of obesity. This finding explains why IR was observed in some non-obese lean sisters and brothers of obese children with IR, indicating IR was the primary disorder[65]. Genes accountable for IR could interact with various environmental factors (such as increased caloric and fat intake augmented with decreased physical activity), resulting in the development of IR, which increases secretory demand on β-cells, causing hyperinsulinemia[66]. Inulin serves as an anabolic hormone responsible for proper nutrient storage following meal ingestion. It also inhibits lipolysis, promotes fat storage, and consequently induces obesity[67]. Certain ethnicities are known to have very high circulating insulin levels, such as Pima Indians, Chinese children, and African American children have a higher prevalence of obesity[68-70]. However, this observation is unique for children and cannot be extended to adults.

Central obesity (with a high waist/hip ratio), a common IR finding, increases the risk for early atherosclerosis, premature coronary artery disease, stroke, and early death. Waist circumference strongly positively correlates with cardiovascular morbidity, BMI, and body fat percentage. The high risk of visceral obesity is due to increased free fatty acid efflux originating from the visceral fat, more glucocorticoid receptors, higher visceral fact concentrations of glucocorticoids, and the low leptin hormone levels with its protective effects compared to the subcutaneous fat[71-73]. As mentioned before, IR increases the risk of obesity, especially the centripetal type, and in turn, central obesity increases the risk of IR and

consequently increases cardiovascular risk. A population-based study by Ikezaki *et al*[74] showed that the reduced cardiovascular risk observed in the Japanese population compared to the American Caucasian population was linked to the considerable population variations in IR.

IR causes many metabolic changes, including hypertriglyceridemia, reduced serum protective high-density lipoproteins (HDL) cholesterol levels, increased atherogenic low-density lipoprotein (LDL) cholesterol particles, and low level of sex hormone-binding globulin (SHBG). Hence, it increases the atherogenic dyslipidemia status and the risk of early atherosclerosis[75]. Hyperinsulinemia also increases renal sodium retention augmented with the IR-induced sympathetic nervous system overactivity and fast vascular smooth muscle growth[76]. IR and hyperinsulinemia also induce early endothelial dysfunction preceding the formation of atherosclerotic plaques, starting the process of atherosclerosis during childhood[77]. These IR-induced changes may precipitate the development of early hypertension. Davis *et al*[78] showed that childhood and current cardiovascular risk factors especially total cholesterol in both males and females and BMI in females, are correlated with a higher carotid intimal-medial thickness in adulthood. In adulthood, coronary artery calcifications are also associated with high blood pressure and reduced protective HDL cholesterol levels measured during childhood. Lipid streaks can be found in the aortic wall in children as young as three years and in the carotid arteries by adolescence[79].

Fatty infiltration of the liver is a common problem observed in patients with IR, which could progress with time to end with inflammation (steatohepatitis), fatty liver fibrosis, and even liver cirrhosis and failure. With the increasing worldwide prevalence of obesity, the non-alcoholic fatty liver has become the most common pediatric liver disease. According to Schwimmer *et al*[80], the rate of fatty liver can reach up to 38% among children with obesity. Peng *et al*[81] showed that the prevalence of non-alcoholic steatohepatitis in children with obesity is strongly linked to high BMI-standard deviation score, gender, uric acid, waist circumference, body fat, IR, and hyperuricemia. Fatty liver infiltration is usually asymptomatic for many years. Still, it can be expected when liver enzymes rise, such as alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transferase, which may indicate hepatic fat accumulation. The aspartate aminotransferase/alanine aminotransferase ratio is generally less than one, but this ratio rises as fibrosis progresses[82]. It is commonly diagnosed as an incidentally by abdominal ultra-sound during an examination for other reasons[83]. Consequently, children with IR and obesity should be monitored to detect liver disease early.

IR and obesity commonly present with pseudo-endocrinal hyperfunction due to a decrease in hormonal binding proteins suggesting a common underlying controlling mechanism. Decreasing cortisol-binding globulin (CBG) causes an increase in free cortisol level, which causes the manifestations of Cushing's syndrome (pseudo Cushing's syndrome), which could overlap with the manifestation of MS. Fernandez-Real showed that the level of CBG is negatively correlated with insulin level[84]. Insulin-like growth factor-I binding protein (IGFBP-1) decreases in IR, causing an increase in free but not the total IGF-I. The increase in free IGF-1enhances the glucose-reducing effect of insulin, causing microvascular complications and manifestations of pseudo-acromegaly (acromegaloidism) with linear and acral growth (acromegaloid features) and signs suggestive of excess GH and normal levels of GH and total IGF-1[85]. IGFBP-1 Levels are negatively correlated with the severity of IR, while IGFBP-3 Levels correlate directly with hyperinsulinism[86]. Hyperinsulinemia also enhances linear growth by upgrading the skeletal IGF-1receptors, augmented by the increased free IGF-1 action. Pseudoacromegaly can also result from ghrelin gene polymorphism, which can induce obesity and IR[87].

IR also causes a reduction of thyroid-binding globulin plasma levels, confusing with the presence of hypothyroidism, and consequently, unnecessary treatment for hypothyroidism. In the presence of low TBG, we should consider other thyroid function tests, including thyroid-stimulating hormone and total and free thyroxine, and triiodothyronine. On the other hand, detecting hypothyroidism in IR and obesity is crucial. It could be the underlying cause of IR as thyroid hormones have a significant impact on glucose metabolism[88]. SHBG is a hepatic-produced protein that adheres to sex hormones with high specificity and affinity in males and females. SHBG is negatively correlated with fasting insulin levels and BMI. Sørensen *et al*[89] showed that puberty is associated with low SHBG levels, explaining the increased cardiovascular risk during puberty. They also showed that SHBG is a potent predictor of insulin sensitivity and metabolic risk during puberty. Chen *et al*[90] found that SHBG is a significant objective element of IR indices and can be used as an adequate positive indicator for IR in patients with polycystic ovary syndrome (PCOS), especially those who are overweight/obese. Reduced SHBG levels increase the free testosterone available to the tissues leading to manifestations of hyperandrogenism such as hirsutism and acne, even in the presence of normal total testosterone levels. Consequently, it induces progressive ovarian pathology, anovulation, and the characteristics features of PCOS[91].

The increased free androgens increase their aromatization and conversion into estrogens, increasing the incidence of adipo/gynecomastia in male adolescents with more GH production and increasing longitudinal bone growth[92]. Littlejohn *et al*[93] described a series of four girls with severe IR, which showed nearly all the features of pseudo-endocrine hyperfunctions associated with IR. The girls had severe prepubertal obesity due to severe IR, followed by the appearance of early childhood pseudo Cushing's syndrome, then manifestations of pseudo-acromegaly, which herald adolescent polycystic ovary syndrome.

People with overweight or obesity commonly have vitamin D deficiency even in different age groups and ethnicity. As vitamin D is fat-soluble, and there is a marked increase in fat mass in patients with obesity, there is a volumetric dilution causing a relative deficiency. Consequently, people with obesity need higher supplemented doses to maintain normal serum levels of 25-hydroxyvitamin D than people with average weight[94]. Lind *et al*[95] showed that serum levels of 25-OH-vitamin D are negatively correlated with fasting insulin and positively correlated with insulin sensitivity. They also showed that IR is associated with low 25-hydroxyvitamin D3 levels. Vitamin D is essential for the normal secretion of insulin.

Consequently, vitamin D deficiency aggravates the metabolic derangement in IR and obesity. Vitamin D binding protein (VDBP) binds to about 90% of the total vitamin D while only 1% of vitamin D metabolites is present in a free unbound form. VDBP is a macrophage-activating factor with a potent tumor growth inhibitor and strong anticancer activity. Ashraf *et al*[96] showed that VDBP levels are inversely correlated with IR and hyperinsulinemia. Consequently, low vitamin D status is associated with higher risks of several cancers in patients with obesity[97]. Meanwhile, Pratley *et al*[98] showed that VDBP polymorphism is connected to the increased risk of diabetes in Pima Indians.

IR-related obesity characterizes by increased markers of inflammation like CRP and erythrocyte sedimentation rates. The inflammation is more common in girls than boys who have BMI more than 95th percentile[99]. CRP levels correlate significantly with BMI and adipose tissue mass in the young adult population[100]. The visceral obesity, systemic inflammation, and cellular dysfunction associated with IR are significant cardiovascular risk factors. When started in childhood and persist until adulthood, it induces various chronic cardiovascular diseases such as atherosclerosis, systemic hypertension, and coronary artery diseases[101]. However, CRP elevation and degree of inflammation could improve with dietary modification and more grain consumption[102]. As asthma and obesity are associated with systemic inflammation, increased pro-inflammatory state, and the effects of increased leptin levels on Th1 cytokine responses, there is an increase in asthma prevalence among children with obesity, especially during puberty[103]. Castro-Rodríguez *et al*[104] showed that BMI positively correlates with the prevalence of asthma in both boys and girls. They also showed that girls who become overweight or obese between 6 and 11 years are seven times more likely to have asthma at age 11 or 13. Consequently, weight reduction helps to improve pulmonary functions and asthma symptoms and reduce the need to use rescue bronchodilators and the frequency of asthma exacerbations[105]. Obstructive sleep apnea, a common complication of obesity, can increase IR. IR is expected to present when children with obesity have obstructive apnea-hypopnea index ≥ 4.9[106].

High insulin levels in IR stimulate insulin and IGF-1 receptors in human keratinocytes, causing the increased thickness of the stratum corneum with hyperpigmentation in a racially dependent manner (Acanthosis Nigricans). The posterior region of the neck, axillae, antecubital fossae and groins are the most common affected sites. In contrast, other flexural areas, sub-mammary region, umbilicus, elbows, knuckles, and, in extreme cases, the entire skin are less commonly involved. The degree of IR and the insulin blood levels positively correlate with the severity of acanthosis nigricans[107-109]. Özalp Kızılay *et al*[110] showed that IR is more predictive of psychiatric illness than obesity-related metabolic comorbidities. Consequently, it is crucial to assess the presence of psychiatric malfunctioning in obese children, particularly those with IR. We highly recommend routine screening to identify the presence of psychiatric disorders in children with obesity.

CLINICAL PRESENTATION

Appropriate history and comprehensive clinical examination provide a lot of information that helps to diagnose IR.

PATIENT MEDICAL HISTORY

IRS is commonly associated with dyslipidemia, obesity, skin changes, atherosclerosis, hypertension, DM type-II, hyperandrogenism, and polycystic ovarian syndrome. The clinical presentation of IR is variable and depends on its etiology and severity. History can elaborate on the presence of high-risk IR. Maternal history of gestational DM, preeclampsia, or intrauterine growth restriction could expect the development of IR, especially in obese offspring. The large or small birth weight for gestational age is also a significant risk factor for IR. Microcephaly, with head circumference less than the 10th centile at birth, may indicate significant intrauterine growth retardation, which could be a sign of genetic causes of IR, or the growth restriction itself could induce IR[111,112]. It is critical to evaluate preceding anthropometric measurements using appropriate growth charts and give attention to the catch-up growth in smaller babies. Particular attention should be given to recent rapid weight gain, specifically if be associated with dysmorphic features. History of cold intolerance, easy bruising, generalized weakness, and easy fatigability could indicate the presence of other endocrine disorders such as hypothyroidism or Cushing's syndrome[113].

The onset and duration of obesity are also crucial to predict IR's presence and complications. Infants and children who developed obesity and significant weight gain before the age of five and particularly in the 1st year of life are more liable to have genetic causes for IR and obesity. Early development and a longer duration of obesity predict an adverse metabolic profile of the affected child[114]. However, all children with overweight or obesity have IR, and not all children with IR are overweight or obese[115]. The dietary history is also essential considering the overall caloric intake, considering the food elements that significantly impact the weight gain and the metabolic pattern in the child with overweight or obesity. Taking a good dietary history is mandatory to identify the dietary components that could lead to obesity development and, at the same time, can give a clue to improve the metabolic derangement even without significant weight loss[116]. At the same time, the sleeping pattern is equally essential to dietary history. The duration of the sleep, the sleep pattern, and the presence of sleeping disorders should be addressed. OSA is a frequent disorder observed in children with obesity, which further increases IR due to various pathologic mechanisms such as tissue hypoxia and sympathetic activation [56]. Therefore, children with obesity and snore, mainly when mouth breathing, should be screened for the presence of OSA with polysomnography[117].

Good medical history should address the lifestyle, sedentary behavior, and the child's physical activity. With the overuse of the media, especially during the current coronavirus disease 2019 pandemic and spending more and more media time, including television, online teaching, computer gaming, and smartphone use, we expect a significant rise in the rate of obesity and consequently IR [118]. However, any physical activity, even non-weight reducing activity, may provide a beneficial metabolic effect on the body fat composition and improve the general body insulin sensitivity. Therefore, any degree of physical activity should be encouraged[119]. As many medications significantly impact insulin sensitivity, a medication history is mandatory while managing a child with either obesity or suspected IR[120]. We should ask about any medications/drugs that affect appetite, glucose, or lipid metabolism. As mentioned before, some psychotropic medications such as Clozapine, Olanzapine, and Risperidone, corticosteroids, growth hormone therapy, some antihypertensive drugs such as beta-blockers, and diuretics as thiazides, antiepileptics as Valproate, and some common antineoplastic drugs as Tacrolimus, Cyclosporine A, and Sirolimus[121]. As IR has many genetic causes, positive family history of similar conditions, obesity, DM type II, or other forms of metabolic disorder is common.

PHYSICAL EXAMINATION

Adequate physical examination is mandatory as it helps assess the presence and the severity of IR and the underlying cause. General appearance can hint about the underlying lesion, especially in the presence of dysmorphic features and pseudo-acromegalic features (with suppressed GH levels), which could signify the presence of genetic or secondary causes of IR. The anthropometric examination is essential during any child examination, particularly when overweight or obese, is expected. Weight, height, BMI, mid-arm, and waist circumference should be measured and plotted on the appropriate charts and growth curves. Height is measured to the nearest 0.5 cm, while the body mass is measured to the nearest 0.1 kg using a standard stadiometer. The waist circumference is measured using a cloth tape at the end of normal expiration to the nearest 0.1 cm at the midpoint between the uppermost lateral border of the right iliac crest and the lowest rib. Children are considered overweight when their BMIs are higher than the 85th percentile for age and sex, or BMI equals to or more than 25 kg/m^2. They are considered obese with BMIs higher than the 95th percentile or BMI equal to or more than 30 kg/m^2[122]. However, not all children with obesity have IR, but most children with BMIs more than 35-40 kg/m^2 have IR[123]. Fat distribution, especially the abdominal fat, impacts the development of IR and consequent non-alcoholic fatty liver disease in obese children. So, we should evaluate the intraabdominal type (apple-shaped) *vs* peripheral fat (gluteal-femoral, extremity, or pear-shaped) and document waist circumference and waist/hip ratio. The body fat percentage can be assessed using different methods, such as an X-scan bioelectrical body composition analyzer[124]. Tall stature may indicate the presence of underlying endocrine or chromosomal disorders, *e.g.*, Klinefelter syndrome.

The blood pressure is measured using an appropriately sized cuff after at least 5 min of rest, preferably with an automated instrument in a seated position. At least two readings are measured, and the average value is used for analyses and adjusted for age, sex, and height. Occasionally, we may need a 24-h ambulatory blood pressure evaluation. Blood pressure could be high in some endocrine disorders that may induce IR, such as Cushing syndrome. Giordano *et al*[125] found an association between a decrease in nocturnal blood pressure and insulin levels (as a measure of IR), regardless of obesity or diurnal blood pressure levels. The pulse also should be evaluated for any resting tachycardia. Flanagan *et al*[126] found that the insulin sensitivity in the young adult correlated with cardiac sympathovagal balance in males but not in females, suggesting the effect of gender on the autonomic modulation of IR. We should also search for signs of heart failure to rule out obesity-induced cardiomyopathy[127]. The examiner should also ask about any signs of respiratory distress (for underlying bronchial asthma), expiratory wheezing, and snoring, indicating upper airway obstruction and possible OSA.

Abdominal examination is a crucial part of child examination for IR. Abdominal obesity is diagnosed when waist circumference equals to/more than 90th percentile for age and gender. After adjusting for BMI percentile, waist circumference significantly correlates with total and abdominal visceral fat and insulin sensitivity. Some studies revealed that BMI and waist circumference together are superior predictors of metabolic risk than only one of them[128,129]. It is also essential to look for striae and detect organomegaly. Hepatomegaly may present as a sign of congestive heart failure due to obesity-induced cardiomyopathy or steatosis and non-alcoholic steatohepatitis[130]. Abdominal pain could occur as a side effect of metformin treatment.

Skin is commonly affected by IR. Obesity, IR, or DM indicators may include hypo/hyperpigmentation, acanthosis nigricans, abdominal skin striae, skin tags, fatty breast (adipomastia) in males, hirsutism, acne, frontal balding, and signs of virilization in females. Higher insulin levels could associate with premature pubarche. Premature pubarche and virilization in girls are potential antecedents of PCOS. It is due to increased insulin levels with a causal relationship between high insulin levels and hypersecretion of the adrenal and/or ovarian androgens[131]. Acanthosis nigricans is a darkly pigmented, velvety, hyperkeratotic, papillomatous skin lesions in body folds such as the skin of the neck or axilla. The presence of acanthosis nigricans is due to acanthocytes' exposure to hyperinsulinemia, interacting with insulin-like growth factor-1 receptors on these cells[132,133]. Multiple skin tags are more sensitive than acanthosis nigricans in identifying abnormal glucose/insulin metabolism. Multiple skin tags should increase suspicion of increased risk of IR or hyperinsulinemia[134]. Examinations of the extremities for strain and deformities should be done as genu varum and other lower extremity postural defects are common in children with overweight or obesity[135].

LABORATORY DIAGNOSIS OF IR IN CHILDREN

Diagnostic standards for IR in children are not yet established due to various causes, including different techniques to measure IR, insufficient patient size, and lack of adequate longitudinal long-term pediatric studies. Thorough evaluation of impaired sensitivity and responsiveness to the insulin thus needs an assessment of insulin dose-response curves. Hyperinsulinemia is defined when the fasting insulin level is > 15 μU/mL, or peak insulin level is > 150 μU/mL or > 75 μU/mL at 2 h after the oral glucose tolerance test, which may indicate IR[17].

DIRECT MEASURES OF INSULIN SENSITIVITY

Direct measures of insulin sensitivity are valid and reliable for the measurement of insulin sensitivity. They include the hyperinsulinemia euglycemic glucose clamp (HEGC) and the insulin-suppression test (IST). These tests are time-consuming require intravenous infusions and frequent sampling. It is troublesome for participants, is expensive, and needs a research setting.

The HEGC is the gold standard to assess IR. However, the frequently sampled intravenous glucose tolerance test (FSIVGTT), and oral glucose tolerance test (OGTT) are also valid and often used methods as they are more simple and more accessible to be used[16]. In HEGC, the insulin is infused intravenously after overnight fasting at a constant rate (5-120 mU/m^2/min), increasing and maintaining a steady state of systemic insulinemia, which induces increased glucose uptake by the peripheral tissues and suppresses the hepatic glucose production, causing hypoglycemia. Consequently, a bedside glucose analyzer regularly monitors blood glucose levels at 5-10 min. An intravenous glucose 20% infusion at variable rates occurs to maintain (clamp) glucose levels within the normal range to maintain a euglycemic state. The glucose infusion rate is adjusted and directly proportional to insulin sensitivity to maintain the euglycemic state. The more glucose is needed to maintain the euglycemic state, the more the body is sensitive to the insulin effect. We need less glucose infusion to maintain the euglycemic state in IR. Caution should be taken to avoid insulin-induced hypokalaemia, and potassium phosphate infusion should be given to prevent hypokalaemia[136]. This test has the advantage of directly measuring the whole-body tissues' glucose disposal at a certain level of insulinemia but has the disadvantage of technical difficulties[137].

The IST also directly evaluates metabolic insulin sensitivity/resistance to the exogenous insulin after suppressing endogenous insulin production. First, we suppress the endogenous secretion of insulin and glucagon by giving intravenous infusion somatostatin (250 μg/h) or octreotide (25 μg bolus, followed by 0.5 μg/min) after overnight fasting. At the same time, we infuse both insulin (25 mU/m^2/min) and glucose (240 mg/m^2/min) in the same vein. We continuously monitor glucose and insulin from the contralateral arm every 30 min for 150 min, after that, every ten minutes till the end of the third hour. We usually reach the steady-state plasma insulin and steady-state plasma glucose (SSPG) between 150-180 min of the test. We evaluate the sensitivity of the tissue to the exogenous insulin by measuring SSPG levels. The higher the SSPG levels are, the lower the tissue sensitivity to the insulin is, and the lower the SSPG levels are, the higher the insulin sensitivity is[138]. This test provides a highly reproducible direct measure of metabolic actions of insulin and is less technical dependant than HEGC. However, applying

IST in the clinical setting is not practical[139].

INDIRECT MEASURES OF INSULIN SENSITIVITY

Minimal model analysis of FSIVGTT provides an indirect estimate of metabolic insulin sensitivity/resistance, acute insulin response, and disposition indexes. After an overnight fast, we inject a bolus of glucose (0.3 g/kg body weight) by intravenous infusion over 2 min starting at time 0; we collect serial blood samples for glucose and insulin level until three hours after the test. The test assesses insulin sensitivity/resistance by a computed mathematical assessment of glucose and insulin dynamics. It examines the plasma glucose dynamics and the glucose per se to promote its disposal and suppress the hepatic glucose production without an increased insulin effect. It is easier than the glucose clamp method[140].

OGTT is an easy, simple test commonly used in clinical practice, especially during early pregnancy, to diagnose glucose intolerance and type 2 diabetes. However, it tests the glucose tolerance and the ability of the body to dispose of the orally ingested glucose and not IR. We give a standard 75 g of glucose orally after an overnight fast. Then blood samples to determine glucose and insulin levels are taken at 0, 30, 60, and 120 min[141]. OGTT provides the benefits of having fewer blood samples with high correlations with the euglycemic hyperinsulinemic clamp in adult studies but not well studied in the pediatric age[142].

SIMPLE SURROGATE INSULIN SENSITIVITY/RESISTANCE INDEXES

These indexes were created to simplify the measurement of insulin sensitivity. They depend on estimating the fasting blood glucose and insulin levels after overnight fasting. Fasting induces a steady basal state where insulin and glucose plasma levels should be maintained in the normal ranges in a healthy human. So, these indexes reflect the basal insulin secretion by pancreatic β cells and the hepatic insulin sensitivity/resistance. These indexes use a specific mathematical formula that corrects the individual variabilities in glucose and insulin secretion and clearance. However, these indexes are insensitive, lack standardization, and cannot define universal cut-off points for IR[143]. The most common indexes used are the homeostasis model assessment (HOMA), quantitative insulin sensitivity check index (QUICKI), insulin sensitivity index, ISI (0, 120), and adipose tissue IR index (Adipo-IR).

HOMA assumes a feedback loop between pancreatic β-cell and liver. This means that pancreatic β-cell is stimulated by glucose to secrete insulin which in turn stimulates the glucose uptake by the liver and inhibits hepatic glucose production (HGP). In IR, there will be suppression of the HGP. HOMA score is calculated from the following formula: [Fasting glucose (mg/dL) × Fasting insulin (μU/mL)]/405.

An important limitation of the HOMA score is that it indicates the fasting steady-state of pancreatic β-cell and not the actual dynamic state of β-cell Insulin secretion. There is insufficient evidence to support HOMA cut-off values frequently used to identify IR in pediatric studies[144].

QUICKI is also derived from fasting blood glucose and plasma levels. It provides a consistent, reproducible, and precise insulin sensitivity index with outstanding positive predictive value. It uses the following formula: 1/[log of fasting insulin (μU/mL) + log of fasting glucose (mg/dL)].

Adding the log of fasting glucose to the log of fasting insulin provides a reasonable correction and better linear correlation with insulin sensitivity by the HEGC method both in diabetic and non-diabetic patients. It is an appropriate and practical test. It can be used in extensive epidemiological or clinical research studies and help follow changes after therapeutic interventions[145]. ISI (0, 120) is developed by Gutt *et al*[146] and uses the insulin and glucose concentrations both fasting (0 min) and at 120 min post- OGTT. It can screen both obesity and glucose intolerance and correlate well with the euglycemic hyperinsulinemic clamp.

Consequently, it is superior to other indices of insulin sensitivity, such as the HOMA formulae[146]. Adipo-IR is obtained by measuring the fasting level of FFA and insulin. Adipo-IR is well correlated with adipose tissue insulin sensitivity. Adipo-IR is well correlated with and a significant predictor of MS. However, its predictive value is affected by age and physical fitness[147].

IR SCREENING

There is no rationale for screening children for IR, even among children with obesity. Considering that IR in children with obesity increases cardiovascular risks, screening for IR is valid. However, any screening program needs accurate, reliable, easy, and reproducible tests. Screening tests for IR also need to be adjusted for ethnic groups, genders, and pubertal stages. Using lengthy and costly methods such as HEGC or IST is impractical. At the same time, tests that depend on fasting insulin as a screening test are unreliable measures of insulin sensitivity[148].

Meanwhile, there is no definitive recommended pharmacological therapy for isolated IR. Accordingly, it will be wiser to screen and actively manage children with obesity rather than screening for IR[149]. Among the tools that can screen for obesity and IR is ISI (0, 120). It has good predictive value for obesity, IR, DM, and cardiovascular disease (CVD) events. However, it needs more evaluation, particularly in the pediatric age. Rutter *et al*[150] showed that ISI (0, 120) and the MS, not the HOMA-IR index, could independently predict CVD. They also showed that MS might not catch all the CVD risks related to IR. Moreover, Adipo-IR may serve as a useful screening tool to detect IR, especially in those with a high risk of developing DM type-II, even in the absence of clinical risk factors such as obesity or impaired glycemia[25].

PREVENTION OF IR

To prevent future IR, we should target all the factors that could help the development of IR in the future, such as factors that affect fetal growth and development as maternal obesity, pregestational and gestational DM, maternal smoking, especially during pregnancy, maternal undernutrition, and premature delivery[2]. Exclusive breastfeeding until at least four months and continuing until the age of two has a significant impact on reducing child obesity and IR in the future[151,152]. However, there are no sufficient data about the direct effect of breastfeeding in IR prevention. However, its role in obesity prevention is solid. The pancreatic β cells differentiate during fetal life. Still, their maturation and ability to secrete insulin in response to glucose stimulation are modulated during the early postnatal life and modified by the weaning practice[153]. Consequently, proper weaning timing and technique are essential contributors to preventing childhood obesity and IR[154,155]. As antibiotic treatment early in life could increase the risk of obesity, co-administering prebiotic with antibiotics could reduce obesity risk, as demonstrated by Klancic *et al*[156].

Obesity is strongly linked to IR either with a cause-result effect, dietary interventions to prevent obesity could help reduce the prevalence and severity of IR. Increased intake of saturated fat is associated with diminished insulin sensitivity in children. However, we may notice some ethnic differences between children[52]. On the other hand, the intake of a healthy diet low in saturated fat and cholesterol starting at the age of 7 mo is associated with a positive impact on IR at the age of nine[157]. Van Hulst *et al*[158] also showed that reducing saturated fat and raising fruit and vegetable intakes during childhood may enhance insulin sensitivity during puberty. Improving physical fitness in toddlers, preschool, and school children, especially those at high risk for obesity, is an effective preventive way to prevent obesity and IR. Even when not associated with weight reduction, physical activity prevents and even improves IR[159].

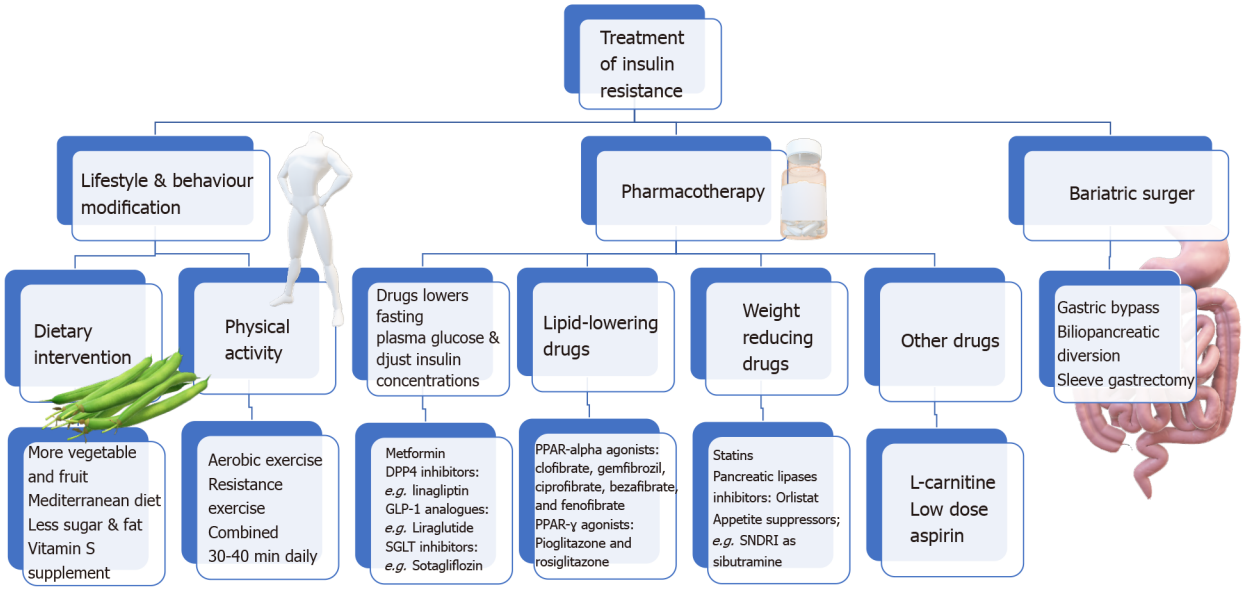
TREATMENT OF IR IN CHILDREN

The main aim of the treatment of IR in children is to prevent the progression of compensated IR to decompensated IR, enhance insulin sensitivity, and treat possible complications. There are three main lines for treatment: Lifestyle and behavior modification, pharmacotherapy, and surgery (Figure 1).

Lifestyle and behavior modification

Lifestyle and behaviour modification is the cornerstone in IR prevention and management. It includes dietary intervention and increasing physical fitness and activity. Exercise may have the upper hand and a more substantial impact in improving insulin sensitivity than the isolated weight reduction[160]. There is a direct relationship between the daily step number the subject does with the blood level of IGF-1 and an inverse relation with high sensitivity CRP. So, physical activity can modulate IR and related inflammation, whereas sedentary time affects fatty acid-binding proteins[161]. Despite the apparent benefits of physical fitness on insulin sensitivity, the exact mechanism is unclear, mainly that improvement in insulin sensitivity occurs earlier than or even without actual loss of body weight[162]. There are not enough studies to compare the different degrees of exercise intensities or the effects of single-session exercise *vs* a training regimen on insulin sensitivity. Also, there is no strong evidence about the optimal exercise form that produces maximum effects on insulin sensitivity. However, a combination of aerobic and resistance exercise training regimens improves insulin sensitivity[163]. Children with IR should be aggressively involved in an exercise program, such as swimming or walking for 30-40 min for most weekdays to provoke glucose entry into the muscles without insulin involvement. Pedometers can be used to monitor their physical activities. Continuation of physical exercise is of utmost importance as cessation of exercise after initial improvement of insulin sensitivity is associated with reverting to the pre-exercise levels (a condition known as rebound phenomenon with higher IR)[164].

Dietary intervention



DOI: 10.4239/wjd.v13.i4.282

Figure 1 Different lines for management of insulin resistance. DPP4: Dipeptidyl peptidase-4 inhibitors; GLP-1: Glucagon-like peptide-1; PPAR: Peroxisome proliferator-activated receptor; SNDRI: Serotonin-norepinephrine-dopamine reuptake inhibitor.

Dietary intervention improves insulin sensitivity in children and adolescents through weight reduction and other unknown mechanisms. Avoiding increasing dietary fat intake, reducing saturated fat intake, increasing unsaturated fat (*e.g.*, olive oil and other vegetable oils) intake, increasing vegetable and fruit consumption, and reducing sugar intake are the main elements for the dietary intervention to improve insulin sensitivity[101]. Adherence to the Mediterranean diet, which incorporates vegetables and olive oil, avoiding the intake of highly processed food and sugar-sweetened beverages, helps to reduce the body weight and improve insulin sensitivity[165-167]. As mentioned before, intake of a high whole-grain diet or dietary fibres improves insulin sensitivity and help to reduce the body weight and BMI in children, adolescents, and adults[168,169]. Probiotic supplementation showed significant improvement in IR indicators in animal studies. It improves inflammatory and oxidative markers, lipid profile, short-chain fatty acids production and microbiota structure. These changes could result from strengthening the intestinal barrier and enhancing the immune system and metabolism. Consequently, adding probiotics to a healthy diet and changing the lifestyle to be more active with/without medications could help to attenuate IR[170,171]. Meanwhile, vitamin D supplementation positively improves insulin sensitivity and cardiovascular and metabolic risk factors in children with obesity[172].

PHARMACOLOGIC MANAGEMENT

There is no specific pharmacologic management for IR. However, pharmacologic treatment is occasionally needed to augment lifestyle management, especially in significant childhood obesity. Because of the severe side effects that could rarely happen, pharmacologic therapy should be sued only in selected cases. We should consider the patient's age, BMI, and associated comorbidities when considering pharmacotherapy. Close monitoring is also required as long-term effects still need more studies[173,174]. Pharmacotherapy involves two main categories: Drugs that decrease fasting plasma glucose and adjust insulin concentrations, lipid-lowering drugs, and drugs that enhance weight loss.

Drugs that decrease fasting plasma glucose and adjust insulin concentrations

The Biguanide-derived metformin is the drug of choice in treating DM type-II in children above ten years. It also showed documented efficacy in improving IR through reducing the body weight, BMI, fasting plasma glucose, and insulin levels. It enhances insulin binding to its receptor even in the presence of receptor autoantibodies (IRS type B) through phosphorylation augmentation and increasing insulin receptor-tyrosine kinase activity[175]. It increases the peripheral tissues glucose utilization by enhancing phosphoinositol 3-kinase at the receptor level, potentiating glucose transporters GLUT1 and GLUT4 isoforms translocation to the cell membrane of various tissues[17]. It is also effective even in the presence of insulin receptor mutation[17]. Recent studies also showed that pre-prandial metformin could acutely reduce blood glucose levels *via* intestinal glucose transport inhibition and increase intestinal glucagon-like peptide-1[176]. It also reduces the food intake with a further reduction of the

body weight, fasting glucose, glycated hemoglobin (HbA1c), insulin, and cholesterol levels. These effects help to improve BMI, body fat composition, lipid profile, and consequently IR. However, although metformin improves insulin sensitivity, it is not indicated in cases with isolated IR[177]. According to the 2017 guidelines endorsed by The Pediatric Society, metformin should be used in selected pediatric patients such as girls with obesity, polycystic ovaries, and glucose intolerance[178]. When using metformin, gradually increased doses can minimize the various gastrointestinal side-effects. Vitamin B12 deficiency could result especially with the long-term use of Metformin, and close monitoring may be required[2].

Incretins are a group of gut-derived metabolic peptide hormones that are promptly secreted in reaction to a meal and promote the reduction of blood glucose levels by augmenting insulin secretion from pancreatic β-cells and inhibiting glucagon release from the alpha cells, a blood glucose-dependent mechanism. There are two main categories of incretins: the intestinal glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide known as gastric inhibitory peptide. Incretins are rapidly deactivated by the enzyme dipeptidyl peptidase-4 (DPP-4)[179]. DPP4 inhibitors such as linagliptin are relatively newly discovered glucose-reducing drugs which antagonize the inhibitory effects of endogenous incretins on insulin secretion, causing increasing insulin secretion in response to blood glucose levels. DPP4 inhibitors improve fasting and post-prandial blood glucose and HbA1c levels[180]. Linagliptin also has protective effects against diabetes-induced macrovascular and microvascular complications. However, Linagliptin is still an investigational drug that has not yet been approved in children and adolescents due to insufficient clinical studies[181].

GLP-1 also reduces the inflammatory cytokine release, inhibits macrophage infiltration into the fatty tissue, the liver, and the vascular wall, and reduces IR-induced chronic inflammation[182]. GLP-1 inhibits food intake through actions in the hypothalamus, including the paraventricular nucleus[183]. Consequently, GLP-1 analogs, such as Liraglutide, could enhance insulin sensitivity and reduce body weight in patients with IR. Danne *et al*[184] showed that Liraglutide use in adolescents with obesity has a safety and tolerability profile like that observed in adults.

Sodium-glucose cotransporters (SGLT) are a group of glucose transporter responsible for apical sodium and glucose transport across cell membranes. They are responsible for the absorption of glucose and galactose in the gastrointestinal tract (SGLT1) and reabsorption of 90% of filtered glucose in proximal renal tubules (SGLT2)[185]. Sotagliflozin (an oral potent dual SGLT1 and SGLT2 inhibitor) effectively improves the glycaemic state by reducing HbA1c, post-prandial blood glucose, body weight in adults with DM type-I and type-II[186]. An animal study showed that the selective SGLT2 inhibitor Empagliflozin was effective as monotherapy or when combined with DPP-4 inhibitor in improving IR in mice with proper glycaemic control[187]. However, we need more consistent data to determine its actual benefits and adverse effects on adults and children with IR.

Lipid-lowering drugs

The PPAR agonists regulate energy (glucose and lipid) metabolism, inflammation, and cell proliferation. They are of three groups: alpha, beta/gamma, and delta used to treat symptoms of MS, primarily by reducing triglycerides and blood sugar[188]. PPARα agonists are the main target of fibrate drugs (clofibrate, gemfibrozil, ciprofibrate, bezafibrate, and fenofibrate). They decrease triglyceride levels *via* PPARα transcription factor, mainly in the liver, inducing fatty acids oxidation, and controlling gluconeogenesis and amino acid metabolism. They are primarily indicated in cholesterol disorders and hypertriglyceridemia[189]. They can also reduce the inflammatory markers such as fibrinogen, CRP, plasminogen activator inhibitor-1, IL-6, and vascular cell adhesion molecule-1 expression[190]. When combined with statins, care should be taken as they may induce a severe form of rhabdomyolysis. Children with severe hypertriglyceridemia (> 400 mg%) can use fibrates to prevent pancreatitis with high tolerability[191]. The PPAR-γ agonists are ligand-activated transcription factors that treat DM and other diseases with IR[192]. PPAR-γ agonists (*e.g.*, pioglitazone and rosiglitazone) can decrease adhesion molecules and inflammatory proteins. They also have lipid-lowering effects through enhancing lipid oxidation, reducing adipocytes' free fatty acid secretion, decreasing intramyocellular lipids, and improving muscular IR. They also decrease 11 beta-hydroxysteroid dehydrogenase type 1 and testosterone levels in IR females[193,194]. Animal studies showed a possible role of PPAR-γ agonists in improving pulmonary inflammation, especially that present in asthma[195].

Weight reducing drugs

Weight reduction by 5%-7% is enough to decrease the diabetes risk by 58% in high-risk persons. Statins are commonly used drugs to reduce body weight. Orlistat is a potent inhibitor of gastric and pancreatic lipase enzymes, y reducing the absorption of cholesterol and triglyceride from the gastrointestinal tract. Orlistat improved lipid profile and led to faster glycaemic control and IR parameters. It also improves retinol-binding protein-4 (RBP-4) and visfatin. RBP-4 is known to be associated with an increased cardiovascular risk. Visfatin is a novel adipokine known to have neuroprotective effects against cerebral ischemic injury[196]. Orlistat can enhance insulin sensitivity in children and adolescents. However, it should be used wisely and in selected cases in this age group[197,198]. Sibutramine is a weight-reducing drug used to treat obesity mainly by its appetite-suppressing effect. Care and awareness about the loss of its effectiveness and the possible detrimental adverse effects should be given[199]. Statins reduce

hepatic cholesterol synthesis by inhibiting the 3-hydroxy-3-methylglutaryl-CoA reductase enzyme, consequently increasing the hepatocyte uptake of LDL decreases the atherosclerosis progression. Even though statins are safe and well-tolerated in children, their long-term safety is not firmly established in this age group[200].

Other drugs

L-carnitine has been used for several years as adjuvant therapy in oxidative stress. A meta-analysis by Xu *et al*[201] showed that L-carnitine is beneficial and effective in treating patients with IR. Children with severe dyslipidemia and IR with a high risk for pancreatitis may get benefit from using daily low dose Acetylsalicylic acid (aspirin 81 mg/d) to inhibit arachidonic acid conversion to prostaglandins G2 and H2, known precursors of thromboxane, and consequently decrease the risk for serious cardiovascular events[202].

SURGERY

Bariatric surgery is presently the most successful approach for sustained and significant weight loss and recovery of the associated comorbidities[203]. Bariatric surgery is beneficial in improving diabetes through the increase in β-cell function and/or mass, increasing insulin secretion, and decreasing IR [204]. Numerous researchers have investigated IR and β-cell function changes after different kinds of bariatric procedures. A meta-analysis by Rao *et al*[205] showed that gastric bypass, biliopancreatic diversion, and sleeve gastrectomy produce an early decrease in IR (within two weeks) through yet unknown mechanisms. Sleeve gastrectomy had an earlier reduction in IR than gastric banding. A Dutch study showed increased acceptance of bariatric surgery by the pediatricians, parents, and adolescents as a therapeutic in children and adolescents with severe obesity who do not respond to lifestyle intervention[206]. However, intestinal bypass surgery in children should possibly only be used in cases of potentially life-threatening complications of obesity such as IR, OSA, dyslipidemia, hypertension, non-alcoholic fatty liver diseases, and bone and joint problems[207].

TREATMENT OF SPECIFIC CASES WITH IR

The treatment can be individualized in certain pathological conditions.

CONGENITAL GENERALIZED LIPODYSTROPHY

Congenital generalized lipodystrophy (CGL) requires multidisciplinary management and should be adjusted according to the specific features of the patients and the severity of the dystrophy. It may involve psychological support, aesthetic surgery, and high carbohydrate and a low-fat diet. Exercise should be tailored according to the type, with regular exercise for type 1 CGL and avoidance of strenuous exercise for type 4. Patients with type 4 may require β-adrenergic blockers or other antiarrhythmic medications. Patients with type 2 CGL and cardiomyopathy should be assessed individually to ensure their fitness for exercise and avoid when needed[208]. The presence of severe hypertriglyceridemia in CGL could benefit from fibrate drugs. Low-dose statins could help to reduce non-HDL cholesterol. If the patients develop DM, Metformin and sulphonylureas are the first lines of therapy. Insulin is usually needed in very high doses[209]. Leptin levels are markedly decreased in patients with generalized lipodystrophy. Leptin analogs as metreleptin can improve metabolic profile in CGL type 1 and type 2. Metreleptin centrally reduces the appetite through its effects on the hypothalamus. Metreleptin has been Food and Drug Administration-approved since 2014 to treat congenital and acquired generalized lipodystrophy with significant improvement of the quality of life and physiological well-being[210].

LEPRECHAUNISM (DONOHUE SYNDROME)

Recombinant IGF-1 is the only treatment available to treat patients with leprechaunism so far through preventing compensatory hyperinsulinemia[211]. IGF-1 has a similar structure to insulin and can reduce blood glucose by 6% of the effect of insulin. It can attach to insulin receptors, enhance peripheral glucose uptake, induce glycogen synthesis, and decrease protein catabolism. The effectiveness of therapy with Recombinant IGF-1 is debatable, and we need further studies. However, evidence from the currently available small number of *in vivo* studies seems promising[212].

HYPERTENSION IN CHILDREN WITH IR AND OBESITY

The presence of hypertension in patients with MS and IR increases the risk of cardiovascular disease and premature death. Angiotensin-converting enzyme inhibitors positively affect hypertriglyceridemia and IR and are considered the first-line drugs in treating hypertension in children with obesity with additional renal and cardiovascular protective benefits[213,214]. However, we need more randomized, controlled, double-blind, and long-term studies for a definitive conclusion.

FATTY LIVER DISEASE

There is no specific pharmacologic treatment for fatty liver disease. The patients should start a low-fat diet and change their lifestyle to a more active style, and be encouraged to exercise. Triglyceride-lowering drugs and antioxidants can also be used. Insulin sensitizers, such as metformin, showed efficacy in animal and human studies[215].

CONCLUSION

As obesity increases in children, IR becomes more prevalent in children and adolescents than before. There is a broad range of genetic and acquired causes of IR. Early recognition of IR in the Pediatric age could prevent many possible short and long-term complications. Both prevention and management of IR resistance in children depend on changing the lifestyle, dietary intervention, and physical modification. Pharmacotherapy is indicated in selected cases. Surgery could help manage specific cases of IR and should be chosen meticulously.

ACKNOWLEDGEMENTS

We thank the anonymous referees for their valuable suggestions.

FOOTNOTES

Author contributions: Al-Biltagi M, Bediwy AS, and Saeed NK collected the data and wrote and revised the manuscript.

Conflict-of-interest statement: No conflict of interest.

Country/Territory of origin: Bahrain

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 308-318

DOI: 10.4239/wjd.v13.i4.308

ISSN 1948-9358 (online)

MINIREVIEWS

Gut microbiota and diabetic kidney diseases: Pathogenesis and therapeutic perspectives

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Qi R, China; Wen XL, China; Xiu Dong, China

Received: May 9, 2021
Peer-review started: May 9, 2021
First decision: July 3, 2021
Revised: August 9, 2021
Accepted: April 2, 2022
Article in press: April 2, 2022
Published online: April 15, 2022

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Abstract

Diabetic kidney disease (DKD) is one of the major chronic complications of diabetes mellitus (DM), as well as a main cause of end-stage renal disease. Over the last few years, substantial research studies have revealed a contributory role of gut microbiota in the process of DM and DKD. Metabolites of gut microbiota like lipopolysaccharide, short-chain fatty acids, and trimethylamine N-oxide are key mediators of microbial–host crosstalk. However, the underlying mechanisms of how gut microbiota influences the onset and progression of DKD are relatively unknown. Besides, strategies to remodel the composition of gut microbiota or to reduce the metabolites of microbiota have been found recently, representing a new potential remedial target for DKD. In this mini-review, we will address the possible contribution of the gut microbiota in the pathogenesis of DKD and its role as a therapeutic target.

Key Words: Diabetes; Gut microbiota; Insulin resistance; Diabetic kidney disease; Pathogenesis; Therapeutic targets

Core Tip: This minireview consolidates the potential role of gut microbiota in the pathogenesis and as a therapeutic target of diabetic kidney disease. It is known that metabolites of gut microbiota such as trimethylamine N-oxide, short-chain fatty acids, and lipopolysaccharides are important mediators of microbial-host crosstalk. However, the main mechanism of how the gut microbiota specifically affects the occurrence and progress of diabetic kidney disease has not yet been fully explored.

Citation: Lin JR, Wang ZT, Sun JJ, Yang YY, Li XX, Wang XR, Shi Y, Zhu YY, Wang RT, Wang MN, Xie FY, Wei P, Liao ZH. Gut microbiota and diabetic kidney diseases: Pathogenesis and therapeutic perspectives. *World J Diabetes* 2022; 13(4): 308-318
URL: https://www.wjgnet.com/1948-9358/full/v13/i4/308.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i4.308

INTRODUCTION

Diabetes mellitus (DM) continues to be one of the most challenging and economically costly diseases in the world, with its prevalence and incidence increasing[1]. About 20%-40% of the affected population will develop into diabetic kidney disease (DKD)[2], which is the primary contributor of end-stage renal disease (ESRD). The global incidence rate of diabetes in 2019 was expected to be 9.3% (463 million people) and may rise to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045[3-5]. The direct health expenses worldwide on diabetes in 2019 were predicted to be USD 760 billion and are projected to increase to 825 billion dollars by 2030 and 845 billion dollars by 2045[6]. The concern is that the prevalence of diabetes might continue to build up due to significantly expanded incidence of childhood obesity.

DKD can occur in type 1 diabetes, type 2 diabetes, and other secondary diabetes. The development of moderately increased albuminuria in patients with type 1 diabetes typically occurs 5 to 15 years after diabetes initiation and progresses through time[7,8]. In a systematic review encompassing nine longitudinal studies of 7938 patients with type 1 diabetes and moderately increased albuminuria, the total incidence rate of moderately increased albuminuria was 28% over the mean 15-year duration of diabetes [9]. In comparison to patients with normoalbuminuria, the relative risk for all-cause mortality was 1.8 (95% confidence interval: 1.5-2.1), with a suggestion of a similar relative risk for cardiovascular mortality[9,10]. Among nearly 5100 patients with type 2 diabetes included in the United Kingdom prospective diabetes study[11], regarding the occurrence and progression of nephropathy, the results are reported as follows: Ten years after the diagnosis of diabetes, the percentages of cases with

moderately elevated, those with severely elevated urine albumin, and those with plasma creatinine concentrations elevated to > 175 μmol/L (2.0 mg/dL) or requiring kidney substitution treatment were 25%, 5%, and 0.8%, respectively.

The human gut nurtures more than 100 trillion microbial cells. The functional gut microbiota serves particular roles in many metabolic aspects of the host, including nutritional metabolism, alloantigen and medicinal metabolism, maintaining the integrity of the intestinal mucosal barricade structure, immune regulation, as well as resistance to pathogens[12]. Microbial cells are susceptibility factors for the development of nephropathy in individuals with a predisposition to nephropathy, such as patients with DM[13,14]. The intestinal flora may influence the development and progression process of DKD by modifying the endocrine functions of the gut and the components of microbial metabolism products, and *vice versa*. Besides, hyperglycemia and progressive kidney disease determine alterations of the gut microbiota[15,16].

In this article, we review the quantitative and qualitative changes in the gut microbiota of DKD patients that lead to this symbiotic disorder and how it contributes to the progression of DKD, and review well-targeted interferences that can reconstruct the symbiotic relationship.

DKD: PATHOGENESIS

DKD is a complicated and miscellaneous disease with numerous interrelated etiologic pathways. Patients with DKD have four main glomerular histopathological changes: Mesangial expansion, glomerular basement membrane (GBM) thickening, podocyte effacement, and glomerular sclerosis. It was believed that these histopathological changes were mainly due to the metabolic and hemodynamic disorders found in diabetes. Hemodynamic derangements are defined as the hyperfiltration which is due to vasoconstriction of efferent arterioles following the activation of renin-angiotensin-aldosterone system (RAAS) under the stimulation of hyperglycemia. Nevertheless, in recent years it has become more and more apparent that despite the irrefutable central role of hyperglycemia in the development of DKD, it is not the only contributor to DKD. In general, the development of DKD involves several pathophysiological pathways including hemodynamic pathways, metabolic pathways, inflammatory pathways, and autophagy pathways.

The changes in renal hemodynamics are partially regulated by vasoactive hormones, especially angiotensin II (Ang II) and endothelin (ET). In cultured rat mesangial cells, glucose increases Ang II production in a concentration-dependent manner, which results in stimulation of transforming growth factor-β1 (TGF-β1) secretion, decreased matrix degradation, and increased matrix accumulation[17]. Temporarily blocking the prediabetic rats' renin–angiotensin system for 7 wk resulted in a sustained reduction in collagen accumulation and gene expression of connective tissue growth factor (CTGF), which mediates downstream events of TGF-β and stimulates fibroblast proliferation and extracellular matrix (ECM) protein synthesis[18,19]. In response to various factors, mesangial cells can release ET-1 and ET receptors, activation of which leads to a complex signaling cascade with resultant stimulation of mesangial cell hypertrophy, proliferation, contraction, and ECM accumulations[20].

The metabolic pathways including four different entities: The polyol pathway, hexosamine pathway, production of advanced glycation end products (AGEs), and activation of protein kinase C (PKC)[21]. Aldose reductase is the first enzyme in the polyol pathway. Studies have shown that the hemodynamic changes caused by early diabetes and the increase in vascular albumin infiltration and urinary albumin excretion (UAE) are phenomena associated with aldose reductase[22]. The hexosamine pathway originates in the third phase of glycolysis, where fructose-6-phosphate is transformed into glucosamine-6-phosphate. Glucosamine-6-phosphate later is utilized as a substrate which augments the transcription of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and TGF-β1[23], which we will discuss in the inflammatory pathways later. Tissue protein glycosylation is also one of the causes of diabetic nephropathy and other microvascular complications. In a long-term hyperglycemia state, part of the excess glucose will bind to free amino acids in the circulation or tissue proteins. The non-enzymatic reaction initially forms reversible early glycosylation products, and then forms irreversible AGEs. Long-term infusion of AGE-albumin to non-diabetic animals led to glomerular enlargement, GBM hyperplasia, mesangial ECM swelling, and albuminuria, which are all consistent with the glomerulopathy analogous to DKD[24]. Hyperglycemia-induced PKC activation in cultured mesangial cells or diabetic glomeruli is associated with a number of aberrations, namely, elevated arachidonic acid secretion and prostaglandins synthesis, elevated expression of fibronectin, α1(IV) collagen, and TGF-β1, and depressed Na+K+-ATPase action[25].

Various growth factors and cytokines may affect renal function directly or indirectly and perform their actions by stimulating other factors. As mentioned before, in cultured mesangial cells, high glucose or Ang-stimulated production of matrix proteins is partially regulated by TGF-β. The mechanisms involve suppression of matrix metalloproteinase synthesis, incentive of metalloproteinase inhibitor production, enhanced CTGF expression, *etc*.[19,26]. The expression of vascular endothelial growth factor (VEGF) is pronounced in quite few cells including glomerular visceral epithelial cells and tubular epithelial cells, where VEGF is able to induce a proliferative and an antiapoptotic response[27]. The

direct evidence that VEGF is a mediator of DKD was collected from research, in which the weight of the kidney, the glomerular volume, the thickness of basement membrane rose while UAE descended in VEGF antibody-treated db/db mice. VEGF antibody administration tended to reduce expansion in total mesangial volume[28]. Each cytokine has several different effects. IL-1 takes a part in the progression of intra-glomerular hemodynamic aberrations associated with prostaglandin production by mesangial cells and can directly increase vascular endothelial cell permeability[29,30]. The expression of renal IL-6 positively correlates with mesangial hyperplasia and tubular atrophy in various kidney disease models [31]. IL-18 triggers the secretion of interferon gamma and results in producing additional inflammatory cytokines including IL-1 and TNF, over-expression of adhesion molecules, as well as inducing endothelial cell apoptosis[32]. TNF is recognized to play a crucial part in the pathogenesis of DKD. TNF is not only cytotoxic to kidney cells, which can induce direct kidney damage, but also involved in processes such as the induction of apoptosis and necrotic cell death[33]. Studies have shown that TNF plays an important part in the progression of kidney hypertrophy and hypofunction, which are the two major changes in the preliminary stages of DKD, indicating that renal level of TNF may even have the potential to be used as a marker for early stage of DKD[34].

Autophagy (originating from the Greek word meaning "self-eating") is a basic cellular process sending intracellular components to lysosomes to be degraded in order to sustain homeostasis and cellular integrality[35]. Podocytes had a high basal level of autophagy. However, diabetic condition *in vivo* and high glucose conditions *in vitro* impaired autophagy, resulting in lysosome dysfunction and apoptosis, as well as autophagy defects leading to podocyte damage[36]. Because the dynamics of the endoplasmic reticulum (ER) appeared to have a crucial function in modulating autophagic fluxes, the cytoprotective capacity of the ER might fail under high glucose-induced unrelieved stress, which causes autophagy disruption, speeding up the deterioration of DKD[37].

The components and activeness of the intestinal flora are symbiotic with the host since birth and are contingent on complex interactions which depend on the host genome, nutrition, and lifestyle. The gut microbiota plays an important role in maintaining the gut in normal individuals and human health as a whole, and its dysfunction is tightly correlated with the occurrence of DM and the progression to DKD. Metabolome-based genome-wide association studies showed that patients with T2DM are distinguished by moderate dysbiosis of the intestinal microflora, for example, by decreased abundance of some prevalent butyrate-producing bacteria, including *Clostridium difficile* SS3/4, *Escherichia coli*, *Prevotella*, *Roscoidium intestinalis*, and *Roscoidium chrysogenum*, as well as by an elevated number of diverse potential pathogens, including *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta*, and *Escherichia coli*, on top of which, there is an enrichment of the identified mucin-degrading species *Akkermansia muciniphila* and sulphate-reducing species *Desulfovibrio sp. 3_1_syn3*[38]. Several pieces of high quality data from the United States Human Microbiome Project (HMP)[39], European Metagenomics of the Human Intestinal Tract (Meta HIT)[40], and several other studies have proven the favorable effects of the balanced intestinal flora on health all the way to the genetic layer, while Tao *et al*[41] revealed that the abundance of the intestinal microflora and the degree of diversity of bacterial groups were significantly different in DM with respect to healthy controls, and DKD with respect to DM. Interestingly, the variables of g_Prevotella_9 (AUC = 0.9) allowed precise identification of DM from age- and sex-matched healthy controls, and the variables of g_Escherichia-Shigella and g_Prevotella_9 (AUC = 0.86) allowed precise identification of DKD from age- and sex-matched DM patients[41].

The gut microbiota participates in the regulation of various host metabolic pathways. Disorders of the gut environment and associated variations in the makeup of the gut microflora, as well as the metabolites produced, represent a condition referred to as “intestinal dysbiosis”[42,43], leading to disorders of interactive host-microbiota metabolism, signal transduction, and immune-inflammatory axes, influence the gut, liver, kidney, muscle, and brain through physiological connection, and thus may trigger a systemic inflammatory response. Under normal circumstances, the gut barricade precludes the transfer of substances and microorganisms from the intracavity to the bloodstream; the gut barricade is composed of distinct constructions/systems: Tight junctions, intestinal epithelial cell membranes, mucus secretion, and immune defensive mechanisms of the gut lining[42,44]. However, intestinal dysbiosis may result in a “leaky gut syndrome”, with increased permeability that enables the leakage of pro-inflammatory bacterial products [*e.g.*, lipopolysaccharide (LPS)], contributing to insulin resistance [45] as well as expediting the development of renal disorders in people with diabetes[14]. Microbial metabolites are essential intermediaries of microbial host crosstalk, engaging in the regulation of host metabolism and gut integrity.

Endotoxin, a phospholipid, is the hydrophobic anchor of LPS which comprises the external layer of the majority of Gram-negative bacteria. Salguero *et al*[46] revealed a significant relevance between the dysbiosis of Gram-negative bacteria which includes increasing relative abundance of *Proteobacteria*, *Verrucomicrobia,* and *Fusobacteria*, raised LPS concentrations, and accumulated state of inflammatory biomarkers consisting of C-reactive protein (CRP), TNF-α, and IL-6 in DKD patients in contrast to the controls[46]. Also, as a result of the leaky gut syndrome, LPS translocation which leads to high circulating levels of LPS, a condition known as “endotoxemia”, stimulates immune system cells, especially macrophages and endothelial cells. In macrophages, LPS activates IL-1R-associated kinase (IRAK) through TLR4-mediated MyD88 and MD2 signaling, with ensuing induction of TNF receptor-

associated factor 6 (TRAF6) binding with IRAK and other proteins forming a large complex, catalyzing the synthesis of a Lys 63-linked polyubiquitin chain of TRAF6 and finally resulting in the activated transcription factor NF-κB and discharged pro-inflammatory cytokines[47], which is known to be important in the pathogenesis of DKD[48].

The hallmark feature of gut dysbiosis is a decrease in the levels of short chain fatty acids (SCFAs)-producing saccharolytic microbes. SCFAs are the end products of fermentation of dietary polysaccharides by intestinal microbiota, including acetate, propionate, butyrate, pentanoic acid, and isobutyric acid[49]. The functions of SCFAs are generally concerned with the activation of transmembrane G protein-coupled receptors (GPR) and the repression of histone acetylation (HDAC)[50], and the increase of glucagon-like peptide-1 (GLP-1) and GLP-2 production through GPR stimulation, along with elevated insulin expression and ensuing augmented insulin sensitivity and proliferation of pancreatic cells. Intriguingly, glucose homeostasis and feelings of satiety are both regulated by gut microbiota components like *Bifidobacterium* and *Lactobacillus* that enhance GLP-1 secretion[51]. Besides, SCFAs can inhibit oxidative stress and inflammation of glomerular mesangial cells induced by high glucose and LPS[52], as well as improve intestinal barrier function[53]. Sodium butyrate treatment markedly reduced the levels of glucose, creatinine, and urea in plasma, attenuated histological changes, involving fibrosis and collagen deposition, and curbed the activity of HDACs, eNOS, iNOS, fibronectin, TGF-β1, NF-κB, apoptosis, and DNA damage in diabetic kidneys[54]. However, not all the remedies of SCFAs showed favorable effects. Lu *et al*[55] discovered aberrant intestinal flora, elevated plasma acetate levels, raised proteinuria, thickened GBM, and loss of renal podocyte foot process in DM rats compared to control rats[55]. In addition, the amount of angiotensin II, angiotensin-converting enzyme, and angiotensin II type1 receptor boosted in DM rats' kidneys, suggesting that redundant acetic acid produced from gut flora disorders may cause kidney damage by activating RAAS in the kidney. It is hypothesized that these differences of SCFAs studies may result from disparate animal models in disparate diseases as well as from the group, concentration, and timing of application of SCFAs.

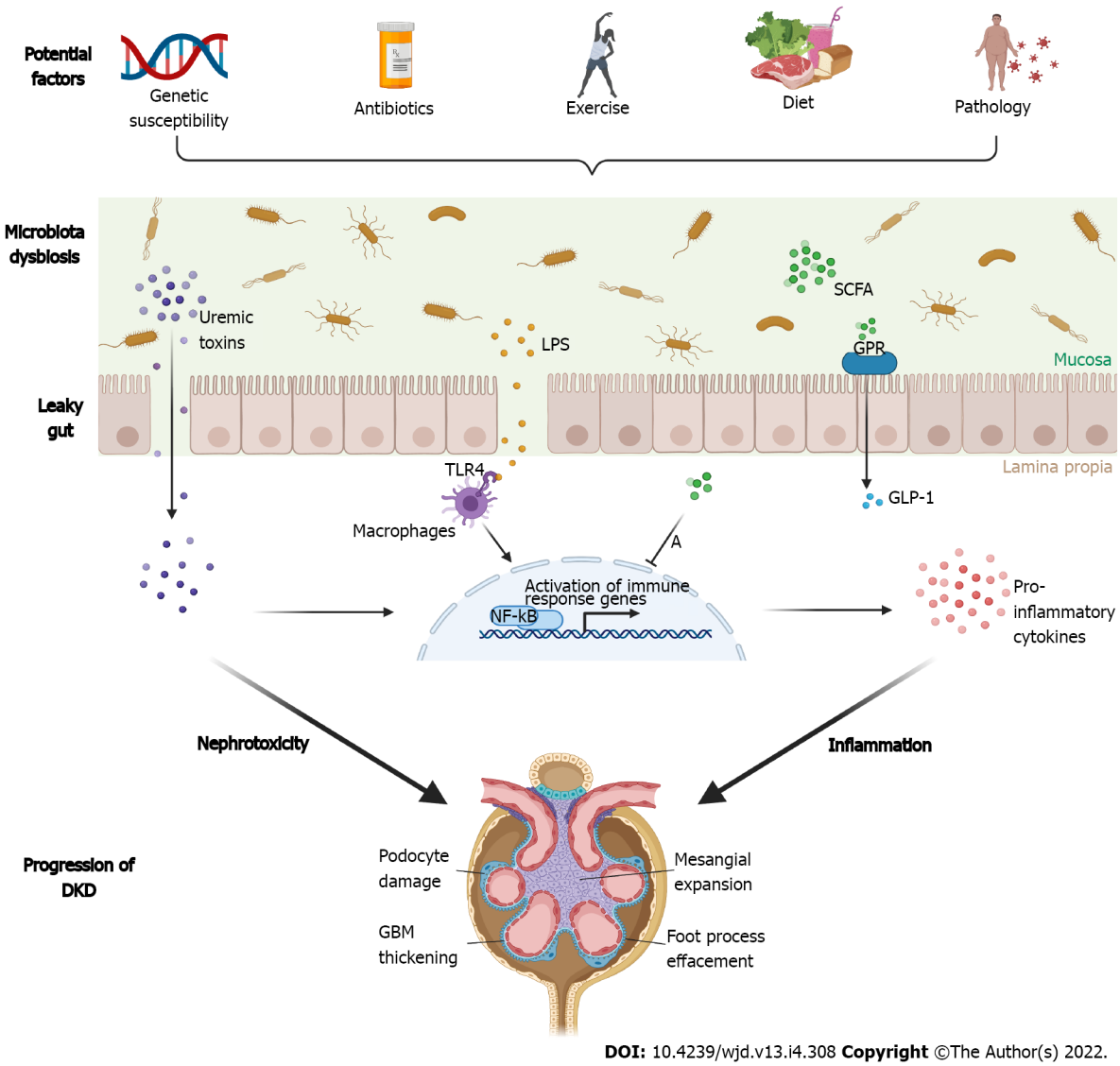
Imbalance of the gut microbiota is also a potential source of uremic toxins. Urea is derived in the liver from the urea cycle and its origin is dietary/endogenous amino acids and their decomposition in the peripheral tissues. The intestinal microbiota uses urease to convert urea into ammonia (NH3) and carbon dioxide. A portion of the ammonia goes through the urea cycle in the liver and is transformed back into urea, whereas the rest of the ammonia is transformed into ammonium hydroxide (NH4OH) and then excreted from the body with feces[13]. Changes in lifestyle and diet as well as reduced fiber consumption can cause imbalance in the intestinal flora and production of an overload of the uremic toxins [*e.g.*, indoxyl sulfate (IS), phenyl sulfate (PS), p-cresyl sulfate (PCS), and trimethylamine-N-oxide (TMAO)]. Normally, the amount of IS receptors [aryl hydrocarbon receptors (AhRs)] may modulate podocyte functionality. Nevertheless, under conditions of imbalanced intestinal flora, AhRs are prolonged activated by broad exposure to IS, which results in progressive damage of podocytes and glomeruli including altered cell morphology, elevated levels of expression of pro-inflammatory cytokines and chemokines, declined podocyte differentiation, and reduced expression of cytoskeletal proteins[56]. Also, AhR was demonstrated to be able to interact with various signaling molecules such as NF-κB, which is responsible for the upregulation of proinflammatory proteins in uremic conditions [57]. Kikuchi *et al*[58] found that the amount of PS (an intestinal microflora-derived metabolite) increased with advancing diabetes in rats in which the human uremic toxin transporter SLCO4C1 was over-expressed in the kidney, whereas it declined in rats that showed limited proteinuria. In pilot models of DM, the giving of PS triggers albuminuria and podocyte injury. In a cohort of DM patients, PS levels were closely related to the baseline and forecasted advancement of albuminuria in patients with microalbuminuria over 2 years[58]. Figure 1 illustrates the pathogenic associations between gut dysbiotic microbiota and development of DKDs from the gut-kidney axis.

THERAPEUTICS AGAINST GUT MICROBIOTA IN DKD

Exercise is considered to be an important potential factor in modification of gut microbiota, which could have both beneficial and harmful effects under some specific circumstances. Moderate levels of exercise may be able to keep balance of gut microbiota and reduce harmful bacteria in the digestive tract to some extent[59]. However, Cani *et al*[60] found that excessively intensive exercise may lead to increased permeability in the digestive tract[60].

Obviously, the host genome is the main risk determinant for a number of different diseases. Nonetheless, not alike the host genome, the genome of microorganisms in the host can be changed. Through the administration of prebiotics (dietary foods that boost the growth or performance of particular microorganisms), probiotics (live bacteria), synbiotics (mixtures of probiotics and prebiotics), as well as antibiotics, people are able to alter the composition of the intestinal microbiota themselves and thereby modify the resultant metabolites.

Animal studies found that high-fat-fed diabetic mice treated with prebiotics (fructo-oligosaccharides, FOS) not only had higher levels of intestinal *Bifidobacterial* and colonic GLP-1 precursor and reduced endotoxaemia, but also obtained improvement on their glucose tolerance and insulin resistance[61]. This



DOI: 10.4239/wjd.v13.i4.308

Figure 1 Pathogenic associations between gut dysbiotic microbiota and development of diabetic kidney diseases from the gut-kidney axis. On the one hand, endotoxins and uremic toxins accumulate because of gut microbiota dysbiosis and leak into the systemic circulation *via* a damaged gut barrier, which effectuates inflammation and nephrotoxicity. On the other hand, the dysbiotic microbiota results in a decrease of short chain fatty acids (SCFAs)-producing gut microbiota. SCFAs can activate transmembrane G protein-coupled receptors, which further stimulate secretion of glucagon-like peptide-1. In summary, SCFAs production in normal condition stabilizes the blood sugar level and presents protective effects on kidney cells. LPS: Lipopolysaccharides; SCFA: Short chain fatty acids; GPR: G protein-coupled receptors; GLP-1: Glucagon-like peptide-1; NF-κB: Nuclear factor kappa beta; TLR4: Toll-like receptor 4; GBM: Glomerular basement membrane; A: Means inhibition; →: Means activation.

dietary shift method also worked in germ-free mice colonized with a synthetic community where at day 35 (7 d following the change to the FOS diet), there was a distinct decline in *Bacteroides caccae* enrichment and a concurrent increment in *B. caccae* enrichment. Importantly, during the same period, there was a significant reduction in the level of IS in the host, and this decrease remained unchanged after 1 wk, suggesting a steady drop in the production of uremic toxins[62]. Li *et al*[63] reported that when feeding diabetic rats with a high-fiber diet, and feeding diabetic control rats with a normal diet or a zero-fiber diet, the former was less likely to fall into the DKD phase featured with albuminuria, glomerular hypertrophy, podocyte injury, and interstitial fibrosis. Fiber can profitably reshape intestinal microbial ecosystem and improve microecology dysbiosis. For example, fiber allowed growth in density of fecal and systemic SCFAs through stimulating the colonization of SCFA-producing bacteria such as the genera *Prevotella* and *Bifidobacterium*. Besides, fiber may intervene the progression of DKD by diminishing the expression of genes which are responsible for the generation of inflammatory cytokines, chemokines, and fibrosis-promoting proteins. SCFAs were nephroprotective in diabetic mice, providing that GPR43 or GPR109A is present. *In vitro* cellular experiments revealed that SCFAs could regulate hyperglycemia-induced inflammation in renal tubular cells and podocytes[63].

Bohlouli *et al*[64] analyzed data from 340 DKD patients by systematically reviewing and quantitatively synthesizing seven RCTs. They found that probiotics consumption beneficially impact the inflammation and oxidative stress biomarkers by significantly reducing high-sensitivity C-reactive

protein (hs-CRP) and malondialdehyde (MDA) plus increasing glutathione (GSH) and total antioxidant capacity (TAC) in subjects. Yet, probiotics had no remarkable effect on concentrations of nitric oxide (NO). Subgroup analysis indicated that when the probiotic dosage was greater 5 billion CFU *per* day, the total impact of probiotics on serum TAC concentrations was more prominent[64]. Vlachou *et al*[65] concluded that most studies showed the beneficial effects of supplementary probiotics in decreasing inflammation and oxidative stress and improving biomarkers of kidney function in DKD patients, and the majority of microbes applied in the studies were in the genera of *Lactobacillus* and *Bifidobacterium*. Doses varied from 2×10^7 to 6×10^{10} CFU/g. The format of the probiotics differed among projects (capsules, pouches, soy milk, yogurt, and honey)[65]. Probiotics use may also help to reinforce the barrier function, through prevention of dysbiosis and regulation of cytoskeletal and tight junctional protein phosphorylation. Guo *et al*[66] illustrated that *Bifidobacterium infantis* and *Lactobacillus acidophilus* were able to safeguard the gut barrier from irritation by IL-1β and thereby preserve the intestinal permeability to an extent. The mechanism may be that the levels of occluding and claudin-1 were normalized and that the IL-1β-induced NF-κB activation was inhibited in Caco-2 cells[66]. Resta-Lenert and Barrett[67] remarked that when the epithelial cell lines were under the exposure to enteroinvasive *Escherichia coli* (EIEC), the application of *S. thermophilus* and *L acidophilus* could sustain and sometimes even strengthen the structures of cytoskeleton and tight junction proteins[67].

There are relatively few studies on synbiotics in DKD. In a randomized, double-blind and placebo-controlled trial encompassing 81 DM patients, the consumption of synbiotic bread containing *Lactobacillus sporogenes* and inulin caused a marked increment in levels of NO in the blood plasma and a remarkable drop in MDA concentrations compared to the probiotic and control breads. However, probiotic bread intake had no significant influence on the levels of TAC, GSH, and catalase in plasma, liver enzymes, calcium, iron, and magnesium in serum, and blood pressure in contrast to probiotic and control breads[68]. There is another study where patients with ESRD who were undergoing haemodialysis (HD) received synbiotic (*Lactobacillus casei* strain Shirota and *Bifidobacterium breve* strain Yakult as probiotics and galacto-oligosaccharides as prebiotics) for 2 wk. The results of the study demonstrated that p-cresol is a constipation-related uraemic toxin, and the three subjects with the highest serum p-cresol level were diabetic HD patients. The synbiotic regimen regularized defecation habits and reduced serum level of p-cresol in HD patients[69].

Hu *et al*[70] found that depletion of gut microbiota by antibiotics significantly alleviated tubulointerstitial injury, reduced IL-6 concentrations in the blood, and efficiently relieved glycemia in DM rats. Meanwhile, it rescued the increased urine albumin/creatinine ratio and N-acetyl-β-D-glucosidase /creatinine ratio. Intriguingly, in DM rats treated with antibiotics, the levels of acetate in the serum also declined significantly and were positively correlated with kidney cholesterol concentrations[70]. Similar results were found in diabetic rats by using broad-spectrum antibiotics, where not only the majority of the intestinal microbiota was thoroughly killed, but also the concentrations of acetate in plasma were reduced, intrarenal RAAS activation was effectively suppressed, and renal injury was mitigated[55]. Antibiotic therapy is unable to eliminate every microorganism that exists in the intestine of mice; however, it is possible to maintain the microbiome in quite low levels, which is why antibiotic therapy is commonly applied to acquire pseudo-germ-free mice in gut microbiota studies even if the requirement of germ-free mouse maintenance is rigid and difficult to fulfil in most laboratories. Nevertheless, a large amount of antibiotics may damage the kidneys of mice. Moreover, the use of antibiotics alone is not the best solution, because of the possible consequences of microbiome abatement, for example, antibiotic-related pathogen aggression[71]. The sensible application of antibiotics to achieve or enforce selection of strains colonized with specific metabolic traits is likely to present a plan that can achieve shrinkage of toxin production and preservation of many of the microbiota's health benefits.

A more sustained and potent treatment to reconstruct a robust microbiome structure and functionality might include contiguous fecal microbiota transplantation (FMT) originating in healthy donors. Barba *et al*[72] found that FMT from healthy mice improved PCS accumulation, glucose tolerance, and albuminuria[72]. Reconstructing a "healthy microbiota" in patients shows great promise for rebuilding gut, immune, and metabolic homeostasis and it has been tested to be secure and well-tolerated in previous clinical trials[73,74].

CONCLUSION

Gut microbiota serves as a central part as the regulator in metabolic and inflammatory homeostasis, functioning as a link between the host and environmental influences. Constituent of the intestinal microbiota in DKD patients varies from that of the healthy population. Both animal and human studies have confirmed the correlation of gut dysregulation with DKD and associated metabolic disorders. Several studies have shown budding therapeutics against gut microbiota on glucose tolerance, insulin resistance, gut barrier integrity, endotoxaemia, uremic toxin, SCFA, TAC, and so forth, which may breed new methods for the prevention and treatment of DKD and relevant metabolic diseases. Howbeit, which gut microbiota constituents are the causes of renal injury and aberrant glucose metabolism, and which are conservational factors against kidney damage and metabolic disorders, are still being

scrutinized, so the systematic application is not currently recommended for DKD treatment and related metabolic derangement. The dose, time length of treatment, and prolonged outcomes of the utilization of various colonies still call for further investigation; extra searches are demanded before gut microbiota therapies can be judiciously assigned for the treatment or prevention of DKD. Diet modification, lifestyle modification, and control of environmental factors are still pivotal strategies to prevent DKD progression. Our understanding of this gut-kidney crosstalk remains rudimentary, even though there is rapidly accumulating information. Additional work is needed to describe the patho-physiological elements of this interrelationship and to invent new treatments strategies to counteract a detrimental loop of DKD-gut dysbiosis which drives renal disorders to ESRD.

FOOTNOTES

Author contributions: Lin JR generated the figure and wrote the first draft; Wang ZT, Sun JJ, Yang YY, Li XX, Wang XR, Shi Y, Zhu YY, Wang RT, Wang MN, and Xie FY contributed to the writing and editing of the manuscript; Liao ZH accepted the editor invitation, conceptualized the topic, and proofread the manuscript; both Wei P and Liao ZH provided supervision and approved the submission of this minireview.

Conflict-of-interest statement: The authors declare no conflict of interest for this article.

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Corresponding Author's Membership in Professional Societies: American Association for Cancer Research, Associate Member, No. 1075629.

S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 319-337

DOI: 10.4239/wjd.v13.i4.319

ISSN 1948-9358 (online)

MINIREVIEWS

Cognitive disorder and dementia in type 2 diabetes mellitus

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Papazafiropoulou A, Greece; Verma AK, India

Received: May 12, 2021
Peer-review started: May 12, 2021
First decision: June 24, 2021
Revised: July 14, 2021
Accepted: March 17, 2022
Article in press: March 17, 2022
Published online: April 15, 2022

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Abstract

Insulin, a key pleiotropic hormone, regulates metabolism through several signaling pathways in target tissues including skeletal muscle, liver, and brain. In the brain, insulin modulates learning and memory, and impaired insulin signaling is associated with metabolic dysregulation and neurodegenerative diseases. At the receptor level, in aging and Alzheimer's disease (AD) models, the amount of insulin receptors and their functions are decreased. Clinical and animal model studies suggest that memory improvements are due to changes in insulin levels. Furthermore, diabetes mellitus (DM) and insulin resistance are associated with age-related cognitive decline, increased levels of β-amyloid peptide, phosphorylation of tau protein; oxidative stress, pro-inflammatory cytokine production,

and dyslipidemia. Recent evidence shows that deleting brain insulin receptors leads to mild obesity and insulin resistance without influencing brain size and apoptosis development. Conversely, deleting insulin-like growth factor 1 receptor (IGF-1R) affects brain size and development, and contributes to behavior changes. Insulin is synthesized locally in the brain and is released from the neurons. Here, we reviewed proposed pathophysiological hypotheses to explain increased risk of dementia in the presence of DM. Regardless of the exact sequence of events leading to neurodegeneration, there is strong evidence that mitochondrial dysfunction plays a key role in AD and DM. A triple transgenic mouse model of AD showed mitochondrial dysfunction, oxidative stress, and loss of synaptic integrity. These alterations are comparable to those induced in wild-type mice treated with sucrose, which is consistent with the proposal that mitochondrial alterations are associated with DM and contribute to AD development. Alterations in insulin/IGF-1 signaling in DM could lead to mitochondrial dysfunction and low antioxidant capacity of the cell. Thus, insulin/IGF-1 signaling is important for increased neural processing and systemic metabolism, and could be a specific target for therapeutic strategies to decrease alterations associated with age-related cognitive decline.

Key Words: Alzheimer´s disease; Diabetes mellitus; Insulin; Vascular dementia; Cognitive decline

Core Tip: Cognitive disorders and dementia have multiple causes and clinical manifestations. Recently, defects in insulin signaling in the brain have been associated with cognitive disorders and dementia. In this regard, insulin signaling pathways in the brain regulate learning and memory, and modulate peripheral energy metabolism. In this review, the pathophysiological factors involved in cognitive disorders, dementia, and diabetes mellitus, and the link between these disorders, are presented in a summarized manner. Finally, we discuss the role of mitochondrial dysfunction in Alzheimer´s disease and diabetes mellitus.

Citation: Ortiz GG, Huerta M, González-Usigli HA, Torres-Sánchez ED, Delgado-Lara DL, Pacheco-Moisés FP, Mireles-Ramírez MA, Torres-Mendoza BM, Moreno-Cih RI, Velázquez-Brizuela IE. Cognitive disorder and dementia in type 2 diabetes mellitus. *World J Diabetes* 2022; 13(4): 319-337
URL: https://www.wjgnet.com/1948-9358/full/v13/i4/319.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i4.319

INTRODUCTION

Diabetes mellitus type 2 and its complications

According to recent data published by the World Health Organization, diabetes mellitus (DM) affects more than 400 million people worldwide and has a high burden of morbidity and mortality. DM is a multisystemic disease that involves several organs. The complications related to DM are mainly due to a chronic hyperglycemic condition and can be divided into macrovascular and microvascular damage. Macrovascular complications are currently well known and include arteriosclerotic alterations of the large-caliber arteries with progressive reduction in the caliber of the vessels, and the consequential increase in the incidence of myocardial infarctions and cerebrovascular accidents. On the other hand, microvascular complications affect the peripheral circulation and small-caliber vessels, involving the eyes, kidneys, lower extremities, and the central and peripheral nervous system[1]. At the eye level, diabetic retinopathy first causes preproliferative microangiopathy associated with cotton-wool spots and then the formation of microaneurysms and angiogenesis (proliferative microangiopathy), leading to hemorrhages that can produce detachment of the retina itself, causing progressive decrease or acute vision loss and the need to perform various sessions of laser therapy; which, nevertheless, is often not enough, and can lead to progressive, irreversible blindness[2]. At the kidney level, DM leads to alterations in renal function that present with a progressive course according to different stages, which include the appearance, in succession and at different periods of time, of microalbuminuria and proteinuria; and this damage can progress to manifest as chronic renal failure, resulting in uremia, and the need to resort to dialysis[3] (Table 1).

At the level of the nervous system, decompensated DM leads to an alteration in tactile, thermal, and pain sensitivity, and to the transmission of internal muscular impulses from movement. Additionally, peripheral neuropathy can lead to more or less serious lesions of the foot at the beginning, and to paresthesia or a burning or stinging sensation in the extremities[4], until it leads to malfunction of the

Table 1 Type 2 diabetes mellitus and its complications

Major complications of diabetes
Macrovascular (large artery damage)
Ischaemic heart disease
Cerebrovascular disease
Microvascular (peripheral neuropathy and damage to the small vessels)
Retinopathy: Retinal microaneurysm and retinal detachment
Nephropathy: Altered of renal function with microalbuminuria, proteinuria, and progression to chronic renal failure
Neuropathy

vascular and nervous components, and the appearance of ulcers which can complicate with infections, degenerate into gangrene, and cause severe tissue damage that may require amputation[5].

However, what is emerging lately is that DM is also associated with another type of very serious and debilitating complication: dementia. In fact, DM appears to be a risk factor for the development of progressive cognitive impairment as well as vascular-based dementia and Alzheimer's disease (AD)[6]. Several studies have shown that people with type 2 DM (DM-2) have a risk factor for mild cognitive impairment and dementia that is greater than 1.5 times. The evidence was strengthened with the early stages of AD that are characterized by an alteration of energy metabolism, and, in particular, by a modified use of glucose[7]. What has emerged from the various studies is that it is not so much the DM itself that causes dementia, but the alterations in blood glucose levels associated with it; as, in fact, there is a close correlation between the glycated hemoglobin value and the possibility of developing dementia. In particular, the The Action to Control Cardiovascular Risk in Diabetes-Memory in Diabetes trial study (ACCORD-MIND) evaluated the relationship between the level of glycated hemoglobin and fasting blood glucose on the ability to perform four cognitive tests that included: the digit and symbol substitution test[8], the Mini Mental Status Examination, the King Auditory Verbal Learning Test, and the Stroop Test. The data showed that a 1% increase in glycated hemoglobin is associated with a significant reduction in scores on various cognitive function tests, and concluded that elevated glycated hemoglobin levels relates to lower cognitive function. However, unlike glycated hemoglobin, a high fasting blood sugar level was not correlated with a worsening of the test score, which shows that chronic hyperglycemia creates the damage, not a single glycemic spike. Nonetheless, what seems worrisome is the demonstration that excessively high blood glucose values are not necessary in order to have this complication[9].

Glycated hemoglobin levels equal to 7% are a value that many diabetologists consider discretely high, and, in and of themselves, sufficient to cause harm. Several mechanisms have been proposed to explain how decompensated DM can lead to dementia: a first hypothesis is that since elevated glucose levels are associated with a higher prevalence of cardiovascular risk factors and cardiovascular disease, the relationship with dementia could be mediated by the latter; however, this hypothesis is refuted by the fact that this relationship is not attenuated by a reduction in cardiovascular risk. It has also been proposed that chronic exposure of the brain to elevated blood sugar levels can accelerate cognitive decline[10]. Additionally, several post-mortem studies in the brains of people with AD have shown the presence of metabolic oxidation products associated with hyperglycemia. Another possibility is related to the fact that high levels of glycosylated hemoglobin imply an inefficient action or a reduced effect of insulin (more on this later) due to insufficient secretion or reduced activity, or both. There are many insulin receptors (IRs) in the brain, some of which play a role in glucose transport, but many are believed to have a role in cognitive and neurotrophic processes. On this basis, therefore, it is suggested that cognitive impairment is a consequence of a reduced action of insulin in the brain[11]. Other studies suggest that frequent hypoglycemia, which the diabetic patient often suffers, may be the cause of dementia: in a recent and large longitudinal cohort study carried out in elderly patients, the presence of severe hypoglycemic episodes was associated with a 2.4% higher risk per year of developing dementia than in DM-2 without this complication[12]. The mechanism by which this occurs is quite intuitive: hypoglycemia, indeed, seems to directly cause neuronal death, especially in particular areas of the brain that are more vulnerable, such as the hippocampus. It should also be taken into account that the prevalence of dementia is higher not only in diabetics but also in hypertensive and dyslipidemic patients, and in many cases these three mechanisms are enhanced in patients with metabolic syndromes. Treatment with antihypertensive drugs in subjects older than 60 years with isolated systolic hypertension, has been shown to reduce the incidence of dementia. The study data would indicate a 50% reduction in the frequency of dementia from 7.7 to 3.8 cases per 100 patients treated per year (P = 0.05)[13]. Nevertheless, with respect to dyslipidemic subjects, statin intake would not affect the prevalence of dementia. This is what emerged from the results of the PROspective Study of Pravastatin in the Elderly at Risk study (PROSPER), where it was observed that cognitive function declined at the

same rate in both study treatment groups, and no significant differences were observed between the patients in the pravastatin group or the placebo group[14]. Mild cognitive impairment is a risk factor for developing a major cognitive disorder over time, and vascular lesions associated with DM, hypertension, and dyslipidemia can gradually trigger neurodegenerative mechanisms of damage[15]. Major cognitive impairment is a highly disabling medical condition that affects all aspects of patient life and the family environment; and, if we take into account that often the diabetic patient is also hypertensive and dyslipidemic, we understand how the risk of developing dementia is very high in these subjects, especially if the risk factors are not well controlled[16].

DM-2 AND DEMENTIA: "A COMPLICATED AND DANGEROUS RELATIONSHIP"

Although DM affects all age groups, its prevalence is the highest in elderly patients (under 65 years of age): 12% to 25%. Population projections suggest that these rates will double over the next twenty years. The prevalence of dementia, of all etiologies combined, increases exponentially with age, and so it represents a major public health problem with a high probability of worsening in the near future. Therefore, there is a bidirectional association between DM and dementia, each of which increases the risk of "looking" at the other, although we do not know precisely how.

DM-2 AND THE RISK OF DEVELOPING DEMENTIA

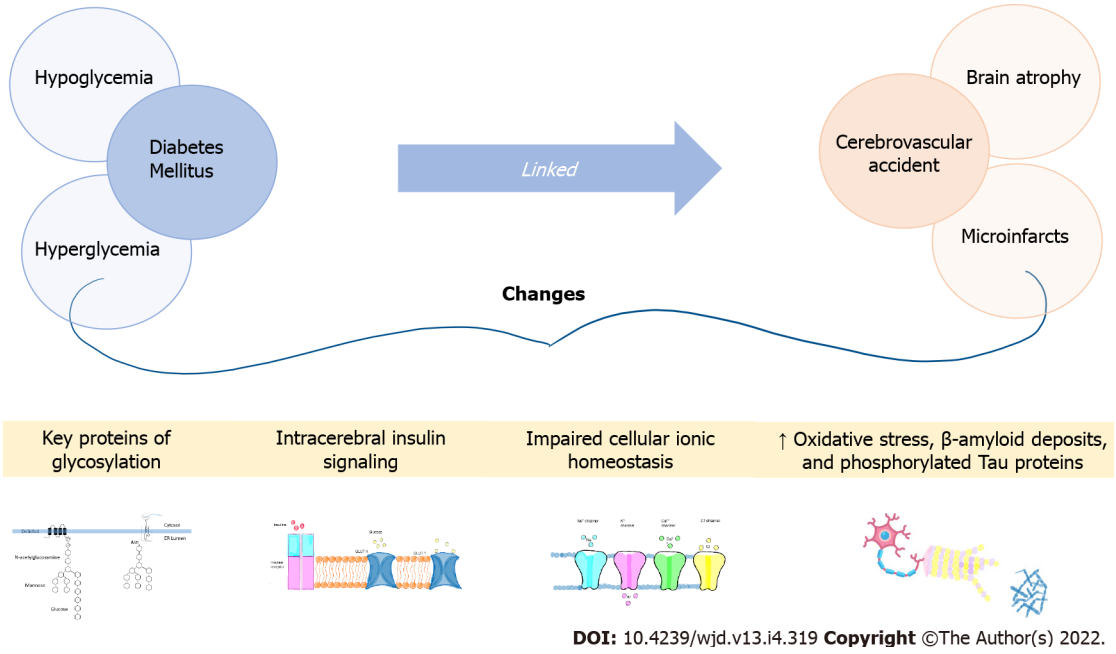
A growing number of studies indicate that patients with DM-2 are between 1.5 and 3 times more likely to develop AD or vascular dementia, and this risk seems especially increased in the group of elderly diabetic patients who have a history of severe hypoglycemia during hospital stays or outpatient management, particularly during multiple hypoglycemic episodes[17]. But, is this increased risk of developing dementia in the presence of DM the result of a causal link? As a matter of fact, cross-sectional and longitudinal studies using brain MRI show an association between DM and the development of brain atrophy, particularly at the level of the hippocampus and the amygdala. These studies also reveal a link between DM and ischemic strokes (cerebrovascular accident) as cortical and subcortical microinfarcts; and, moreover, severe hypoglycemia leads to brain damage, particularly in the cortex and hippocampus[18]. The pathophysiological hypotheses proposed to explain the increased risk of dementia in the presence of DM are diverse. Microinfarctions can result from hypoglycemia or microvascular changes secondary to hyperglycemia, and are one of several factors involved in this pathophysiology[19]. The latter can also lead to changes in key proteins by glycosylation; and, alterations in intracerebral insulin signaling pathways could cause a loss of cellular ionic homeostasis, oxidative stress, an increase in β-amyloid deposits, and phosphorylation of tau proteins (to be discussed later). This last mechanism is cited more frequently to explain the increased risk of dementia, even in the presence of a pathology compatible with AD[20]. Also, DM-2 continues to be an important cardiovascular and cerebrovascular risk factor that increases the risk of stroke and, ultimately, of vascular or mixed dementia, with an overlapping presentation of both being frequent (15% to 20% of dementias)[21] (Figure 1).

VASCULAR DEMENTIA

First of all, dementia is defined as a decrease in cognitive functions (attention, memory, judgment, *etc.*) that leads to a significant loss of autonomy in the affected person; where cognitive impairment and loss of autonomy exceed what is expected in normal aging. The term 'vascular dementia' is used when cognitive and functional losses are related to damage to the cerebral vascular network, in other words damage to the vessels that circulate blood in the brain[22]. Keep in mind that we do not always talk about dementia when there is a cognitive disorder. Instead, when the cognitive difficulties are mild, and the autonomy of the person is not compromised, we use the term 'mild cognitive disorder of vascular origin' according to the DSM-V. Mild vascular cognitive disorder can progress to vascular dementia (major cognitive impairment) but it does not necessarily do so; thus, the importance of understanding what is happening in order to intervene appropriately[23].

DIFFERENCES BETWEEN VASCULAR DEMENTIA, AD, AND MIXED DEMENTIA

Vascular dementia and AD are two types of dementia. They share similarities but differ in terms of causes, the course of the disease, and the type of impairment of cognitive abilities, among other things. The cause of AD is still unknown. However, it is known to be associated with the death of neurons in certain areas of the brain. The affected areas present characteristic abnormalities (neuritic plaques and



DOI: 10.4239/wjd.v13.i4.319

Figure 1 Diabetes Mellitus and its association with Dementia. The alteration in the peripheric and central glucose levels increases the risk of cerebrovascular accidents due to different causes associated with vascular or mixed dementia. The figure has been designed using some resources from Falticon.com.

neurofibrillary tangles)[24,25]. In general, the areas involved in memory are affected first, then the damage gradually spreads to other areas of the brain[25]. In vascular dementia, it is the cerebral vascular network that is involved and there may be a multi-infarct vascular dementia (2 or more simultaneous or asynchronous arterial or venous territorial ischemic vascular events), a vascular dementia as a sequel to a heart attack or hemorrhage in some strategic topography, or a mild or greater cognitive impairment associated with confluent and progressive subcortical lesions of the cerebral microcirculation. This vascular network refers to everything that has to do with blood vessels including veins, arteries, and capillaries. Since the vessels that can be affected are numerous, the clinical picture is more variable than in AD. Here, it is not necessarily the areas responsible for memory that are initially affected[22].

Studies of the brains of patients who died of AD and had vascular dementia showed that the signs of both diseases were often found simultaneously. The researchers concluded that both causes could contribute to the clinically observed difficulties, which leads us to then speak of mixed dementia (AD + vascular cognitive decline)[26] (Table 2).

CAUSES OF VASCULAR DEMENTIA

The brain feeds on oxygen and glucose transported in the blood by the cerebral vascular network, which is formed by a multitude of blood vessels that cover all parts of the brain; and the malfunction of these vessels can have the effect of depriving oxygen to specific areas of the brain, therefore weakening or destroying neurons in these areas[27]. Various problems can affect the vascular network and its many blood vessels, such as the narrowing, blockage, or rupture of arteries or veins. In these cases, the blood flow that ensures the survival of the cells is disturbed and the health of the latter is seen threatened. The involvement of the cerebral vascular network is also usually influenced and accompanied by other diseases like atherosclerosis, DM, or arterial hypertension[22,28].

COGNITIVE DISORDERS ASSOCIATED WITH VASCULAR ALTERATIONS

The cognitive disorders found in people with vascular damage vary depending on the nature of the problem (narrowing, obstruction, bleeding), the type of vessels affected (small or large vessels), and the location in the brain (forward or backward); on the surface or in the center, and on the right side or on the left side. Therefore, the symptoms can be very varied and affect both cognitive functions (attention, reasoning, language, *etc.*) as well as motor and sensory functions[22,29]. Nevertheless, some disorders in diseases of a vascular nature are more common than others. Among the most common symptoms found

Table 2 Difference between vascular dementia, Alzheimer dementia, and mixed dementia

	Characteristics
Vascular dementia	Cerebral vascular network involving veins, arteries, capillaries.Clinical presentation is more variable
Alzheimer dementia	Caused by the death of nerve cells (neurons) in certain selected areas of the brain. Characteristic abnormalities (neuritic plaques and neurofibrillary tangles)
	Memory impairment at initial clinical presentation.
Mixed dementia	Vascular disease + Alzheimer's disease

are slower processing of information, difficulty with cognitive flexibility, or frequent need for help to remember the information learned. These disorders are also associated with changes in mood, slowed motor skills, increased fatigue, and sometimes a less confident gait associated with postural instability [23]. Some people present a dysexecutive problem with reduced speed of the thought process, attention, working memory, the ability to solve problems and make decisions, making mistakes in terms of daily activities with problems in performing simultaneous, dual or multi tasks, and sometimes failure to remember recent information[30].

DIABETES AND AD EPIDEMIOLOGY

Longitudinal studies have shown that the cognitive decline in patients with DM-2 is up to two times faster than in physiological aging, and that diabetic patients are at increased risk of mild cognitive impairment[31,32]. In addition, a pioneering study in the 1990s, the Rotterdam study, investigated the link between DM-2 and different types of dementia including AD. They showed that DM-2 nearly doubled the risk of dementia, with the strongest relationship being to vascular dementia but also observed with AD[33]. The relationship between the accumulation of vascular risk factors (DM, high blood pressure, heart disease, and smoking) and AD showed that DM and smoking were the most important risk factors, and that AD risk associated with DM, regardless of other vascular risk factors, was higher than previously reported[34]. Since then, numerous longitudinal studies have been conducted. Most have identified DM as a risk factor for AD[35]. Studies that specifically analyze the incidence of dementia in DM-2, after adjusting for glycemic control, microvascular complications, comorbidities, and high blood pressure and cerebrovascular accidents, have also shown to have a higher risk. Eight of the thirteen population-based longitudinal studies analyzed found an increased risk of AD in adults with DM, ranging from 50% to 100%[19]. These results were confirmed by two large population studies with a 10-year follow-up[36,37]. Poor glycemic control and duration of DM were later identified as risk factors for AD[38-40]. In a meta-analysis, with a total of 6184 people with DM and 38,530 non-diabetics, the relative risk of AD for people with DM was 1.5 (95% confidence interval: 1.2-1.8)[41]. A meta-analysis involving 1746777 subjects found similar results with a relative risk of AD in DM of 1.53 (95% confidence interval: 1.42-1.63)[42]. Other studies have linked various forms of peripheral insulin resistance[43], such as prediabetes[44], metabolic syndrome[45], obesity induced by a high-fat diet[46], and non-alcoholic fatty liver disease[47], with AD and cognitive impairment (Table 3).

PATHOPHYSIOLOGY

Neurodegenerative diseases such as AD are predominantly sporadic, and the underlying pathophysiological mechanisms are not yet fully understood. Although there are obvious differences between AD and DM, they share pathophysiological mechanisms such as: protein aggregation, mitochondrial dysfunction, chronic inflammatory response, apoptosis, synaptic failure, and decreased neurogenesis. As previously mentioned, numerous epidemiological studies have shown a link between neurodegenerative diseases and DM-2, but the pathophysiological link that unites them remains to be discovered. Even if the vascular hypothesis is widely suggested to explain the possible influence of DM-2 in AD[48-50], the hypothesis most studied at present is that of the implication of the insulin signaling defect which is a fundamental characteristic of DM-2.

There is evidence in favor of the implication of a defect in insulin/insulin-like growth factor 1 (IGF-1) signaling in AD[51,52]. As indicated below, altered insulin signaling influences the pathogenesis of DM-2 and AD. This shows its role in neurodegenerative processes and the probable implication of other pathophysiological mechanisms common to metabolic disorders and neurodegeneration.

Table 3 Summary of studies relating diabetes and Alzheimer's disease

Ref.	Study	*n*	Conclusion
Ott *et al*[33], 1999	Prospective population-based cohort	6370 older adults	Diabetes mellitus almost doubled the risk of dementia. Patients treated with insulin were at highest risk of dementia
Luchsinger *et al*[34], 2005	Longitudinal	1138 older adults	Four risk factors (diabetes, hypertension, heart disease, and current smoking) were associated with a higher risk of AD. The risk of AD increased with the number of vascular risk factors. Diabetes and current smoking were the strongest risk factors
Xu *et al*[38], 2009	Population-based cohort study	1248 dementia-free cohort	Uncontrolled diabetes increases the risk of Alzheimer's disease and VD. Their findings suggest a direct link between glucose dysregulation and neurodegeneration
Wang *et al* [36], 2012	Population-based cohort study	615529 diabetic patients; 614871 random controls	Diabetes may increase the risk of AD in both sexes and all ages
Tolppanen *et al*[40], 2013	Case-Control study	3012 diabetic patients; 3372 AD	Individuals with clinically verified AD are more likely to have a history of clinically verified and medically treated diabetes than the general aged population, although the difference is small
Huang *et al* [37], 2014	Population-based cohort study	1000000 random controls; 71433 diabetic patients	Newly diagnosed DM was associated with an increased risk of AD. The use of hypoglycemic agents did not ameliorate the risk
Gudala *et al* [35], 2013	Meta-analysis	28 studies	The results showed a 73% increased risk of all type of demetia, 56% increase of AD, and 127% increase of VD in diabetes patients
Biessels *et al* [19], 2006	Meta-analysis	14 studies	There is convincing evidence that shows an increased risk of dementia in people with diabetes. The risk factors and mechanisms that drive the association between diabetes and accelerated cognitive decline and dementia need to be identified before adequate treatment measures can be developed
Cheng *et al* [41], 2012	Meta-analysis	19 studies	Diabetes was a risk factor for incident dementia (including AD, VD, and any dementia) and MCI
Zhang *et al* [42], 2017	Meta-analysis	17 studies	The risk of AD is higher among people with diabetes than in the general population

DM: Diabetes mellitus; AD: Alzheimer's disease; VD: Vascular dementia; MCI: Mild cognitive impairment.

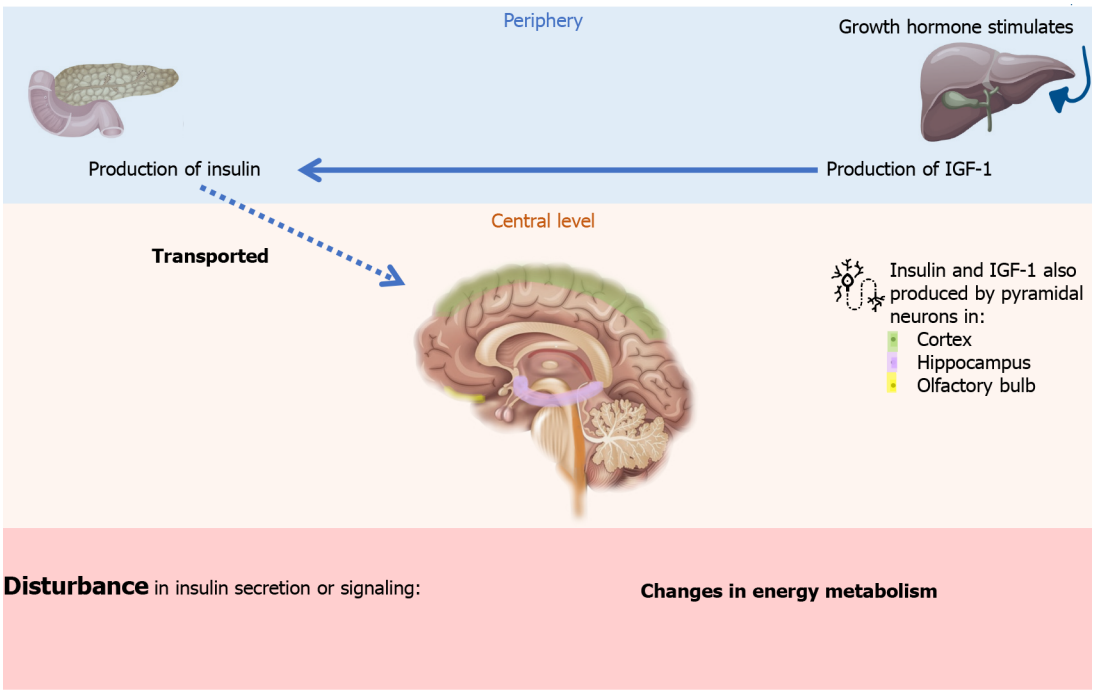
THE INSULIN/IGF-1 SIGNALING PATHWAY

Insulin and the IGF-1 are polypeptidic hormones that are very similar in structure and function. Insulin is mainly secreted by the pancreas when blood sugar is perceived to be high, while IGF-1 is mainly secreted by the liver[53,54]. However, these two molecules are also found in the brain[55]. Historically, insulin is considered a peripherally-secreted anabolic hormone that plays a role in the storage and use of metabolic reserves. Insulin also exerts pleiotropic functions on protein metabolism (increased synthesis and inhibition of proteolysis), growth, control of apoptosis, and development. The most studied role of insulin is that it plays a part in glucose homeostasis and energy balance. These peripheral actions are controlled (at the brain level) by neurons, known as glucose-sensitive, that are present in the hypothalamus. Hence, insulin regulates body weight, energy homeostasis, and peripheral lipid and glucose and protein metabolism. Therefore, a defect in insulin secretion or signaling at the periphery or at the central level could cause changes in energy metabolism throughout the body, including the brain [54].

The source of insulin in the brain is still under discussion. It is thought that insulin from pancreatic β cells is transported to the brain through the blood-brain barrier (BBB) by a saturable process *via* receptors. However, insulin and IGF-1 are also produced by pyramidal neurons in the cortex, hippocampus, and olfactory bulb. Unlike peripheral tissues, brain insulin does not have a direct influence on glucose uptake in neurons, but plays a central role in modulating many functions in the brain and, in general, through effectors posterior, promotes cell survival (Figure 2).

The effects of insulin result from its binding to a specific membrane receptor, the IR, and are expressed primarily in its three target tissues: liver, muscle, and adipose tissue. However, this receptor is also present on nerve endings in key brain regions such as the olfactory bulb, hypothalamus, cerebral cortex; cerebellum, and hippocampus. It should be noted that there is a striking similarity between the IR and the IGF-1 receptor (IGF-1R) in various brain regions, which could lead to an overlap of signal transduction pathways and to the same neuronal effects[56,57].

Two pathways are involved in intracellular events after the binding of insulin and IGF-1 at its receptor and the activation of its tyrosine kinase function of its intracellular domain: the Shc proteins (Src homologous and collagen protein) that activate the MAP kinase (mitogen-activated protein) pathway, resulting in the translocation of extracellular signal-regulated protein kinases (ERK) to the



DOI: 10.4239/wjd.v13.i4.319

Figure 2 Insulin and insulin-like growth factor 1 production. Both can be produced at the periphery and transported to the brain through the blood-brain barrier, but pyramidal neurons can also produce them. The figure has been designed using some resources from Freepik.com and Flaticon.com. IGF-1: Insulin-like growth factor 1.

nucleus and the effects of mitogenic insulin; and, on the other hand, the IRS proteins (IR substrate 1 and 2), which activate the phosphatidylinositol-3 kinase (PI3K)/AKT (also called protein kinase B) pathway upon which the metabolic effects depend. Conversely, the phosphorylation of IRS proteins on serine residues by protein kinases c-JUN kinases (JNK), atypical protein kinases C (PKC), and inhibitor of nuclear factor kβ kinase (IKKβ), plays an antagonistic role to that of the phosphorylation of only tyrosine residues, and causes the dissociation of IRS proteins from the IR or the IGF-1 receptor, and promotes their degradation; thus inhibiting the downstream insulin/IGF-1 signaling. This suggests that in maintaining the protein stability, IRS acts as a critical point in the insulin signaling pathway, and can determine the extent of its actions. In addition, negative control of the insulin signal may come from the degradation of the hormone by the insulin-degrading enzyme (IDE), or by dephosphorylation of the receptor or IRS proteins by tyrosine phosphatases PTPases such as PTP1B and LAR (a molecule related to the common antigen of leukocytes)[57,58].

Other mechanisms are also involved in the negative regulation of the insulin signal: dephosphorylation of the phosphoinositides by lipid phosphatases such as PTEN (homolog of phosphatase and tensin deleted on chromosome ten) and SHIP (Src homology 2 containing inositol 5-phosphatase domain) invert the PI3 kinase signal. The PI3K/AKT pathway can modulate several downstream effectors including glycogen synthase kinase-3β (GSK-3β), mTOR (mammalian target of rapamycin) kinase, Caspase-9, and the FoxO1 transcription factor (Forkhead box protein O1). These different posterior effectors regulate a variety of important functions that are commonly disrupted in neurodegenerative disease such as apoptosis, autophagy, inflammation; nerve cell metabolism, protein synthesis, and synaptic plasticity. So, it is not surprising that insulin signaling has finally been shown to improve neuronal survival[59,60].

ROLE OF THE INSULIN/IGF-1 SIGNALING DEFECT

With normal aging, there is a gradual loss of regulation of insulin secretion leading to hyperinsulinism as well as decreased expression and function of IRs. Decreased insulin expression, reduced delivery of insulin to the brain, and low binding affinity of insulin for its receptors, lead to a state of insulin resistance in the brain. Interestingly, this physiological decrease in insulin signaling seems more marked in neurodegenerative diseases such as AD[61].

ROLE OF INSULIN RESISTANCE IN AD

Several studies have reported that peripheral insulin resistance can promote the onset of AD by reducing insulin supply to the brain and increasing levels of amyloid beta (Aβ), phosphorylation of tau protein, oxidative stress; pro-inflammatory cytokines, production of advanced glycation products (AGEs), advanced glycation end products; dyslipidemia, and apoptosis[62]. However, it has been proposed that insulin resistance is not limited to peripheral tissues, and, in particular, that the brain itself could become insulin resistant with or without the presence of DM-2, and that this could promote (or even lead to) the appearance of key pathophysiological factors of the disease[11,60]. Some researchers have even used the term "type 3 diabetes" to explain these phenomena[60]. But this new definition is questioned by other authors who prefer the term "insulin resistant brain state"[11,63].

Likewise, several insulin signaling markers have been found in the brain of AD patients[60,63], and the selective increase in brain insulin of patients with AD or high risk of AD, by intranasal insulin administration, resulted in improved memory functions[64].

When examining the existence of brain insulin resistance in patients with AD and mild cognitive disorder, it has been shown that the hippocampus, and to a lesser extent the cerebellar cortex, of patients with AD show a reduction in the insulin signaling pathway by IR -> IRS-1 -> PI3K -> AKT and IGF-1 by IGF-1R -> IRS -2 -> PI3K, compared to healthy tissue. This dysfunction occurred independently of diabetic status and APOE ε4 genotype, and gradually worsened as the AD progressed; furthermore, there was a reduced activation of this same pathway when comparing patients with established AD with healthy controls, despite increasing the insulin dose tenfold[11]. These results were associated with elevated levels of phosphorylated IRS-1 proteins at serine residues 636 and 616 (IRS-1 pSer) (which inhibit insulin signaling). Other studies have also shown elevated levels of the IRS-1 proteins pSer312 and pSer616 in association with neuronal insulin resistance in AD[63], leading some authors to suggest that the detection of elevated protein levels of IRS-1 phosphorylated on serine residues could serve as a potential biomarker of neuronal insulin resistance in AD, as is already the case for insulin resistance in peripheral tissues.

The participation of brain insulin resistance in AD has been demonstrated but the obligatory questions are: Is the observed insulin resistance due to inherent resistance of IRs, or to alterations in the transit of insulin across the BBB? Is the involvement of insulin resistance in AD primary (isolated manifestation with selective brain damage) or secondary (after systemic insulin resistance due to obesity, DM, nonalcoholic fatty liver disease, or metabolic syndrome)?

Or, we should consider insulin resistance diseases as processes that can affect one or more organs and tissues, in the same way that atherosclerosis can affect one or more vessels, and produce different manifestations of the disease. On the other hand, there are mechanisms of oxidative stress and chronic inflammation that are associated with clinical processes like DM[49].

PROTEIN AGGREGATION IN AD: B-AMYLOID PEPTIDE AND TAU PROTEIN

β-Amyloid peptide

The term β-amyloid peptide refers to a collection of peptides of 39 to 43 amino acids in length that are formed by cleavage of the amyloid precursor protein (APP) under the action of β and γ secretases. They are products of normal cellular metabolism and probably have a physiological role that is not yet fully understood. The abnormal oligomerization of some of these peptides (such as Aβ-42) and the formation of extracellular plaques containing Aβ fibrils (neuritic plaques) in their center, constitute one of the histopathological markers of AD in post-mortem brain tissue. In sporadic AD, part of the oligomerization of Aβ may be caused by decreased degradation and elimination of Aβ from the brain. The Aβ can be degraded by various peptidases such as IDE, neprisilin, angiotensin-converting enzyme, and many serine proteases. Recent *in vivo* studies have shown that insulin resistance can contribute to amyloid deposits in the frontal and temporal areas in asymptomatic subjects[50].

Experimentally, the induction of insulin resistance in rats increases the production of Aβ by increasing the activation of β-secretase and γ-secretase, and by decreasing the levels of the IDE[46]. Insulin and IGF-1, by activating the PI3K/MAPK pathways, stimulate the transport and clearance of Aβ out of the central nervous system by increasing the expression of Aβ transporters in cerebrospinal fluid. On the other hand, the accumulation of Aβ could also be explained by the effect of insulin on IDE. The main function of IDE is to break down insulin; however, Aβ is also a substrate for IDE, but with lower affinity. Therefore, when insulin rises, it inhibits the breakdown of Aβ by IDE; nevertheless, Aβ can be degraded by others[65].

The activity of IDE decreases with age and there is a reduction in its activity similarly in patients and experimental models of AD[65]. Thus, insulin resistance through decreased IDE activity or, to a lesser extent, competitive inhibition of IDE by insulin during prolonged hyperinsulinism, could lead to defects of IDE autophagy, and, consequently, to a decrease in the turnover and/or neutralization of amyloidogenic proteins in β cells, as well as a defect in the degradation of Aβ that would promote AD-related neuropathological lesions[65]. Therefore, insulin resistance is believed to play a role in the accumulation

of Aß, one of the key markers of the pathogenesis of DM-2.

Tau protein

Tau is a protein that belongs to MAPs (microtubule-associated proteins). In its primary conformation, the tau protein is a soluble and unfolded protein that participates in the stabilization of microtubules and in the axonal growth of neurons. In AD, however, tau is hyperphosphorylated due to inappropriate activation of several proline-directed kinases, including glycogen synthase kinase-3 beta (GSK3β). This results in the folding of the tau protein and self-aggregation into insoluble fibrillar fiber structures (paired helical filaments and straight filaments) that form neurofibrillary tangles, dystrophic neurites, and neuropil threads[66].

Insulin regulates GSK3β; therefore, insulin and IGF-1, by inhibiting GSK3β, inhibit the phosphorylation of tau, and enhance its binding to microtubules. Insulin manages the balance of tau phosphorylation through subsequent activation and inactivation of GSK3β through the Pi3K/Akt signaling pathway[66]. The GSK3β is one of the major signaling molecules downstream of Akt, and the insulin signaling defect in obesity and DM-2 results in aberrant activation of GSK3β leading to increased phosphorylation and accumulation of tau. Therefore, the molecular pathways of DM and AD run parallel in the pathogenesis of these diseases, and the Pi3K/Akt signaling pathway plays a critical role in the neuropathology of tau. Likewise, the alteration of glucose metabolism due to insulin resistance can affect the pathology of tau through the dysregulation of O-linked-N-acetylglucosaminylation (O-GlcNAcylation)[66].

Like phosphorylation, O-GlcNAcylation is a dynamic post-translational modification that involves the attachment of N-acetyl-d-glucosamine (GlcNAc) residues to the hydroxyl group of serine and threonine residues, and it is deregulated in obesity and DM-2. Decreased brain glucose metabolism and O-GlcNAcylation have been shown to lead to hyperphosphorylation of tau in both *in vivo* and *in vitro* models. By contrast, increased O-GlcNAcylation prevents the pathological accumulation of tau. Moreover, in experimental models, the specific elimination of insulin in the neurons of NIRKO (Neuronal Insulin Receptor Knockout) mice leads to hyperphosphorylation of tau associated with a decrease in phosphorylation of Akt and GSK3β. Similarly, the insulin signaling defect in IRS-2 knockout (KO) mice results in the accumulation of hyperphosphorylated tau protein. This accumulation has been attributed to protein phosphatase 2a (PP2A), an enzyme responsible for the dephosphorylation of tau. However, a decrease in GSK3β dephosphorylation was found in this same animal model, suggesting that GSK3β, again, could be responsible for the accumulation of hyperphosphorylated tau[67].

The patterns of tau phosphorylation between NIRKO mice and IRS-2 KO mice vary, suggesting that tau phosphorylation could be controlled not only by insulin resistance but also by other factors such as hyperinsulinism, hyperglycemia, and inflammation. Increased insoluble hyperphosphorylated tau protein and deposition of neurofibrillary tangles occur in various animal models of obesity, DM-2, or AD, with altered insulin signaling. Therefore, altered insulin signaling could promote the formation of neurofibrillary degeneration, disrupt neural cytoskeletal networks and axonal transport, and lead to loss of synaptic connections and progressive neurodegeneration[68] (Figure 3).

These results suggest that insulin resistance accelerates the onset and increases severity of AD, particularly in situations that predispose to the development of tau disease. Furthermore, the increase in the cytosol of IRS-1 pS312 and pS616 is consistent with the presence of neurofibrillary degeneration in the brains of AD patients, whereas pS312 is limited to the nuclear region of cells in controls. These findings suggest that phosphorylated IRS-1 species may cause tau pathology in AD, beyond their role in the development of brain insulin resistance[69].

The combination of all the aforementioned elements described, demonstrate the effects that the insulin/IGF-1 signaling defect could have on tau aggregation in AD.

BRAIN GLUCOSE METABOLISM

There may also be a relevant relationship between neurodegeneration, insulin signaling, and glucose utilization in the brain. Glucose metabolism and insulin signaling are essential for normal brain function, and circulating glucose levels play an important role in learning and memory functions.

Brain glucose metabolism in AD

One of the pathological features of AD is the extreme drop in energy metabolism in the affected areas of the brain. Regional patterns of brain hypometabolism are seen in the early stage of AD. Glucose is necessary for the synthesis of several neurotransmitters such as acetylcholine, dopamine, γ-aminobutyric acid (GABA), and glutamate, *etc*., which mainly participate in synaptic plasticity and cognitive functions[70]. However, cerebral glucose hypometabolism is not directly related to insulin resistance in the brain since, unlike in the periphery, this resistance does not affect neuronal glucose uptake[11]. Rather, glucose hypometabolism in AD may be the result of reduced postsynaptic neurotransmission (a likely effect of reduced insulin signaling in the brain). This is because glutamate and other depolarizing agents stimulate glucose uptake in the brain and the potency of this effect is

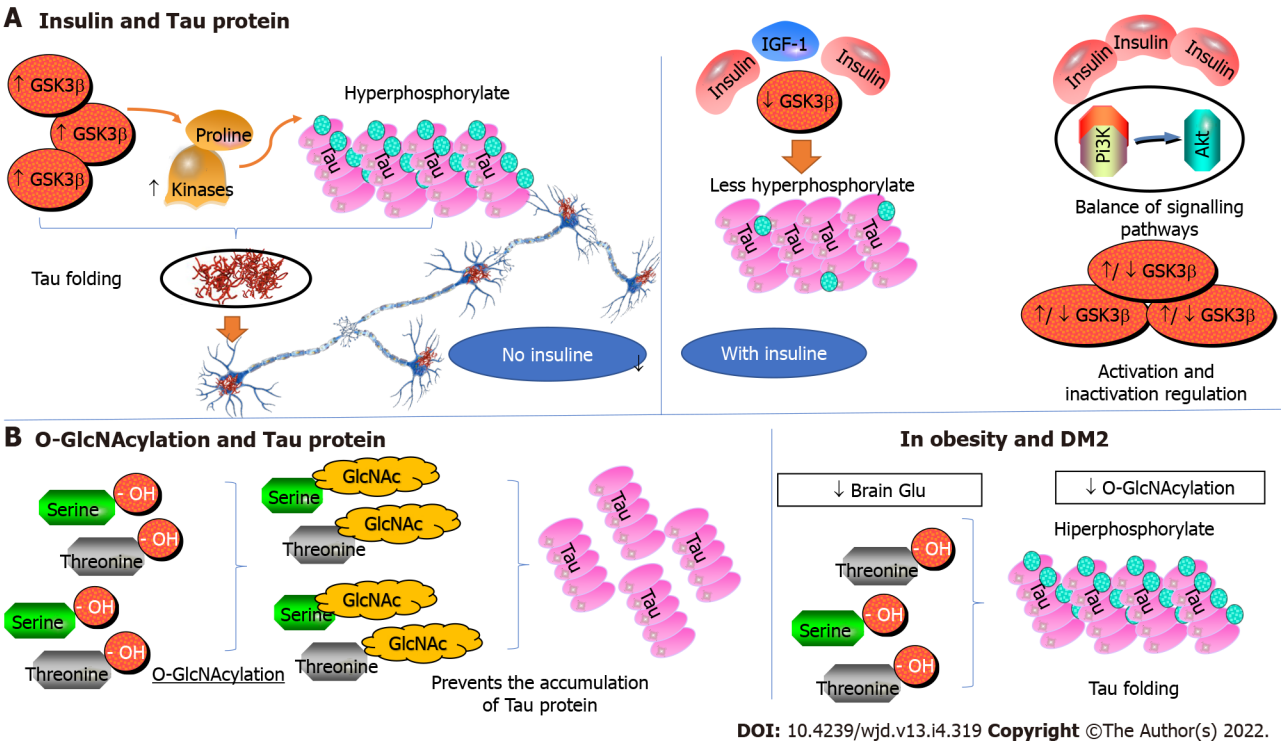


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Figure 3 Tau protein regulation. A: Insulin and Tau protein with no insulin increase hyperphosphorylate of Tau folding the protein; B: O-GlcNAcylation and Tau protein can regulate glucose amination to serine and threonine decrease the formation of Tau folding. Some pictures were taken from Qiagen Pathways. GSK3: Glycogen synthase kinase-3; IGF-1: Insulin-like growth factor 1; Pi3K: Phosphoinositide-3 kinase; Akt: Serine/threonine specific protein kinase; GlcNAc: O-GlcNAcylation.

reduced in AD[11].

NEUROINFLAMMATION IN AD

Numerous studies have shown the presence of inflammatory markers in the post-mortem brains and blood of AD patients. There is considerable evidence to suggest that inflammation modulates cognition; from post mortem studies, to analyses of genomic association, to *in vivo* models addressing its use (for example, of chronic injections of lipopolysaccharide (used to induce inflammation) that accelerates the progression of AD), or from human studies showing that patients with systemic infection have greater cognitive impairment. The induction of metabolic diseases in rodents also induces neuroinflammation: mice fed a high-fat diet showed a higher expression of inflammatory markers in their brains, and cognitive impairment[71]. Furthermore, hyperinsulinism associated with insulin resistance leads to the production of cytokines in the central nervous system[71]. The tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), which are pro-inflammatory cytokines, are known to activate the nuclear factor kappa B (NF-κB) pathway that leads to the transcription of pro-inflammatory genes that exacerbate this cycle. Thus, chronic inflammation may represent an underlying mechanism common to AD and metabolic disorders (Figure 4).

SYNAPTIC TRANSMISSION AND MEMORY

The role of insulin in modulating cognition is suggested in part by the high density of IRs in the hippocampus, cortex, and amygdala, and by the fact that these receptors increase in response to spatial memory training[72]. Furthermore, the acute administration of insulin improves memory performance in rats, and improves verbal memory, attention, and cognition in humans, by activating IRs in the hippocampus. Nevertheless, there is conflicting evidence from studies using NIRKO mice (transgenic mice in which the IR gene has been deleted in the brain). NIRKO mice do not show alterations in memory performance, suggesting that other mechanisms may also contribute[72]. However, NIRKO mice have increased glycogen synthase kinase 3 beta (GSK3-beta) activity, resulting in hyperphosphorylation of tau protein[72]. Conversely, a growing body of evidence indicates that insulin plays an important role in cognition, and although the underlying molecular mechanisms remain unclear,

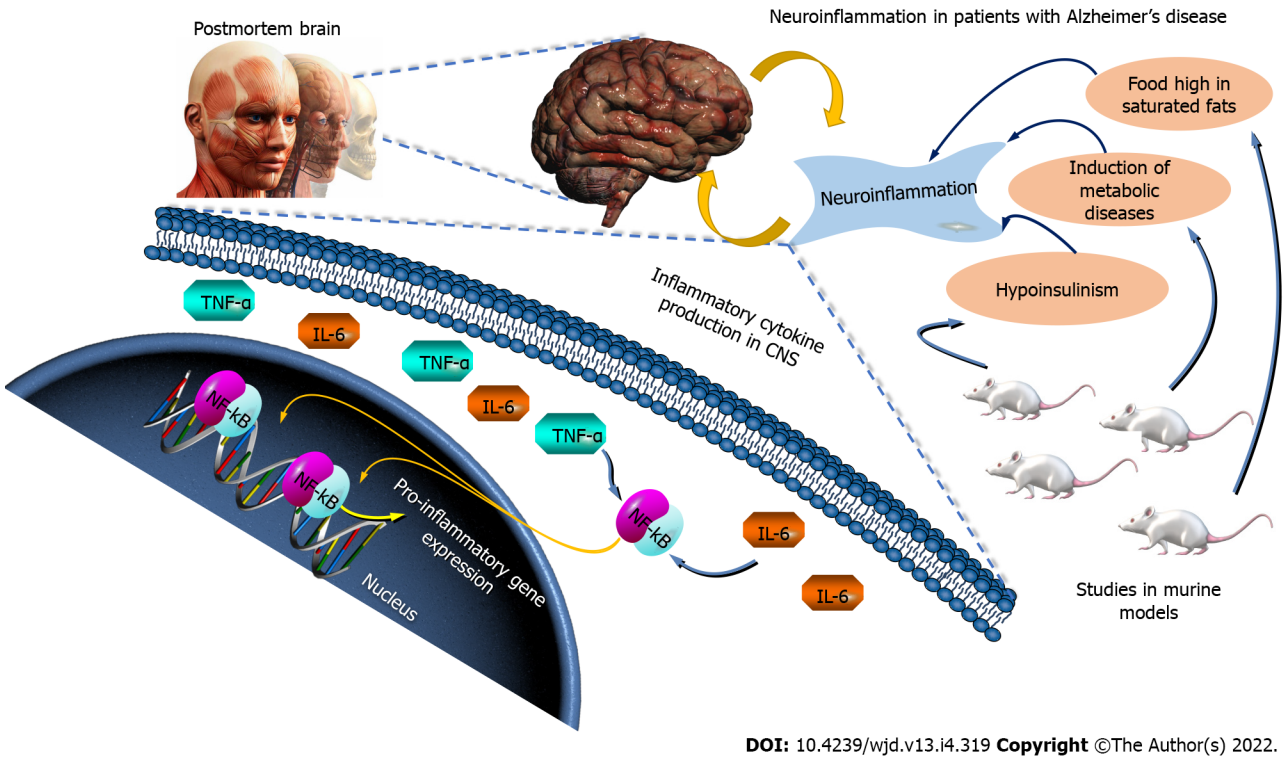

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Figure 4 Neuroinflammation in patients with Alzheimer's disease. Inflammation leads to the production of inflammation cytokines in central nervous system, activating x that impacts the transcription of pro-inflammatory genes. Some pictures were taken from Qiagen Pathways. TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin 6; NF-κB: Nuclear factor kappa beta; CNS: Central nervous system.

studies indicate that insulin resistance in the hippocampus is a risk factor for cognitive decline and dementia in AD[73].

The effects of insulin on cognition appear to depend on the activation of the PI3K/MAPK pathways, through their ability to modulate synaptic plasticity, synaptic density, neurotransmission, and possibly neurogenesis. In addition, insulin induces or modulates a series of neurotransmitters that are part of learning and memory, such as acetylcholine. Activation of the PI3K/MAPK/ERK pathways also serves the effect of glutamate and g-aminobutyric acid receptors. They also enhance protein synthesis and maintain dendritic spine stabilization, which has been shown to be essential for long-term potentiation (LTP) in the hippocampus, and for memory consolidation[74] (Figure 5).

INSULIN RESISTANCE: CAUSE OR CONSEQUENCE OF AD?

A study in a population with AD found that up to 80% of patients had DM-2 or insulin resistance, suggesting that AD may lead to the manifestation of DM. Unfortunately, there are no longitudinal studies that demonstrate whether the presentation of DM occurs after the onset of AD or if it precedes the AD diagnosis. Tissue analysis confirms that insulin resistance in the brain deteriorates with the progression of AD, but when it appears in the progression of the disease is unknown. Additionally, studies have shown that Aβ can also affect insulin signaling in multiple ways[52]: Competing with or reducing the affinity of insulin for its own receptor; Inhibiting IR autophosphorylation and subsequent activation of PI3K/Akt; Causing inhibitory phosphorylation on a serine residue of IRS by stimulation of the N-terminal kinase c-Jun (JNK); and Inhibiting the activation of Akt preventing its interaction with isoenzyme 1 of the pyruvate dehydrogenase-lipoamide kinase (PDK1).

The Aβ derivatives (ADDLs) induce abnormal IR expression and disrupt insulin signaling, thus potentially contributing to the development of central insulin resistance. There is also the question of the involvement of tau protein in insulin resistance, as researchers very recently demonstrated that mice deficient in tau protein develop an abnormal response to insulin in the hippocampus. The question now is whether an abnormal tau protein (such as the hyperphosphorylated tau protein in AD) could have the same impact on brain insulin resistance[75].

If the faulty insulin/IGF1 pathway leads to inflammation, it has also been proposed that neuroinflammation in AD may lead to faulty insulin signaling in neurons. For example, inflammation associated with peripheral infections produces a peripheral inflammatory response that, in turn, can reach the brain without knowing by what process.

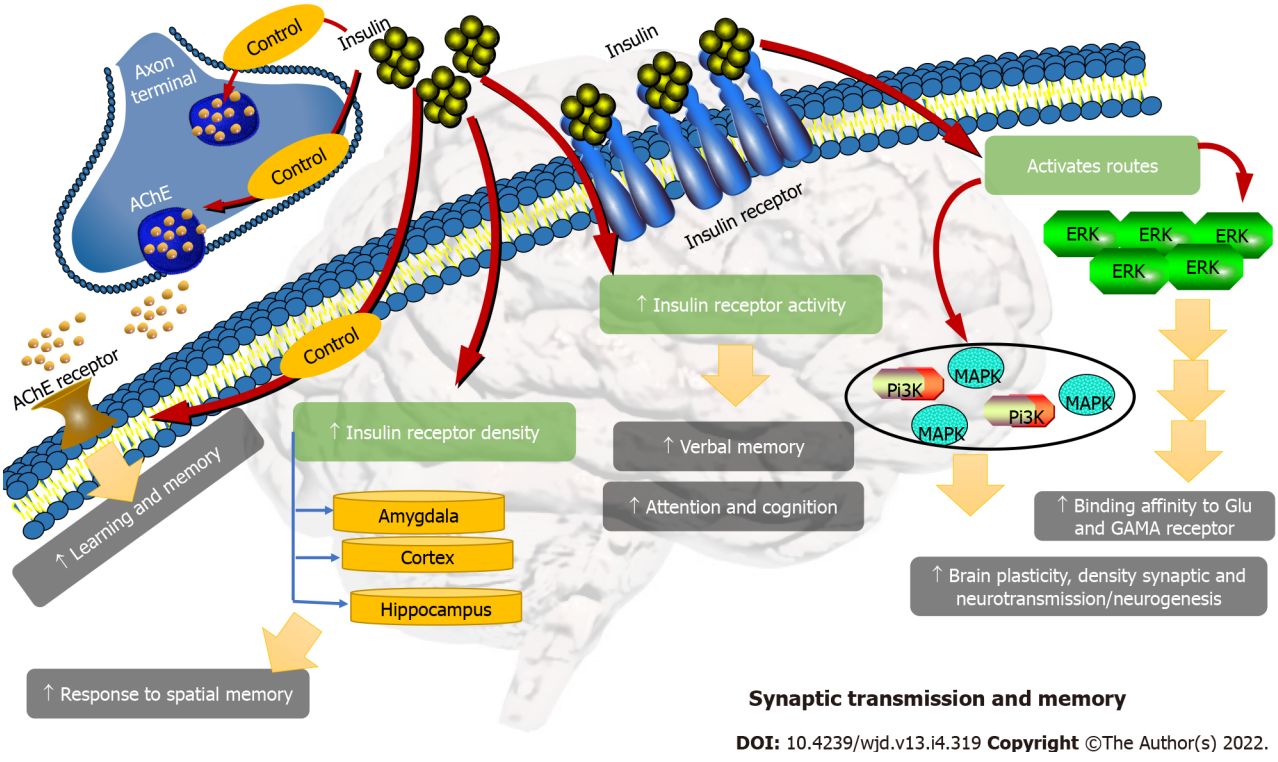

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Figure 5 Synaptic transmission and memory. Insulin increases the density of insulin receptors, its activity, the activation of routes such as phosphoinositide-3 kinase, mitogen-activated protein kinase, and extracellular signal-regulated kinase and regulates the binding of acetylcholine with its receptors, modulating cognition, memory, neurotransmission, and neurogenesis. Some pictures were taken from Qiagen Pathways. AChE: Acetylcholine; Pi3K: Phosphoinositide-3 kinase; MAPK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase.

At the molecular and cellular level, when cytokine receptors (such as TNF-α) are activated, cellular stress pathways (JNK and IKK) that cause phosphorylation at a serine residue of IRS-1 are activated, and thus inhibit intracellular signaling of insulin in the brain. The TNF-α is secreted in the brain in response to infections and abnormal protein aggregation, and is increased in the cerebrospinal fluid of AD patients and transgenic mouse models. Initial evidence linking inflammatory pathways and the disruption of insulin/IGF1 signaling in AD, comes from the observation that Aβ induces IRS-1 inhibition through the TNF-α[75] and the removal of IRs from cell membranes. Based on this evidence, it seems plausible to postulate that inflammation plays a role in disrupting insulin/IGF1 signaling pathways in AD and metabolic disorders[76] (Figure 6).

AMYLIN AS A LINK BETWEEN DM-2 AND NEURODEGENERATION: AD

Amylin polypeptide is a pancreatic hormone that is elevated as a crucial component in the etiology of DM-2 and AD[77]. Recent studies have shown that people with DM-2 are twice as likely as the rest of the population to suffer from AD, which is the leading cause of dementia worldwide. Therefore, there must be a biological process that facilitates the development of neurodegeneration in insulin resistant patients. These two pathologies, as we know, have some points in common: Both are particularly favored by obesity; They are also associated with groups of proteins from the same family (that of amyloids); In the case of DM, it is amylin (an endogenous protein with a hyperglycemic effect) that accumulates in the pancreas; In AD, it is β-amyloids that accumulate in plaques between neurons.

However, these two peptides have been found in the pancreas of people with DM and their presence seems to coincide with the progression of the disease. Amylin is a hormonal peptide released by pancreatic beta cells just like insulin. The coexistence of β-amyloid, tau protein, and amylin in the brain and pancreas, demonstrates their ability to promote amyloid accumulation and cell dysfunction, neuronal dysfunction, and pancreatic β-amyloid accumulation. That is, β-amyloid protein, tau protein, and amylin can interact synergistically, increasing to promote amyloid deposition, oxidative stress, mitochondrial dysfunction; inflammation, insulin resistance, and cell damage, culminating in dysregulation of amyloid metabolism, and glucose and neurodegeneration in AD and DM-2. In previous studies, it has been found that high plasma amylin concentration is associated with the incidence of AD, which suggests that amylin is a risk factor for AD[78,79]; and, amylin deposits in the brain have been found to interact with the β-amyloid protein and with tau protein in the pancreas and the hippocampus, which provides new evidence of a potential overlap between the understanding of the mechanisms of

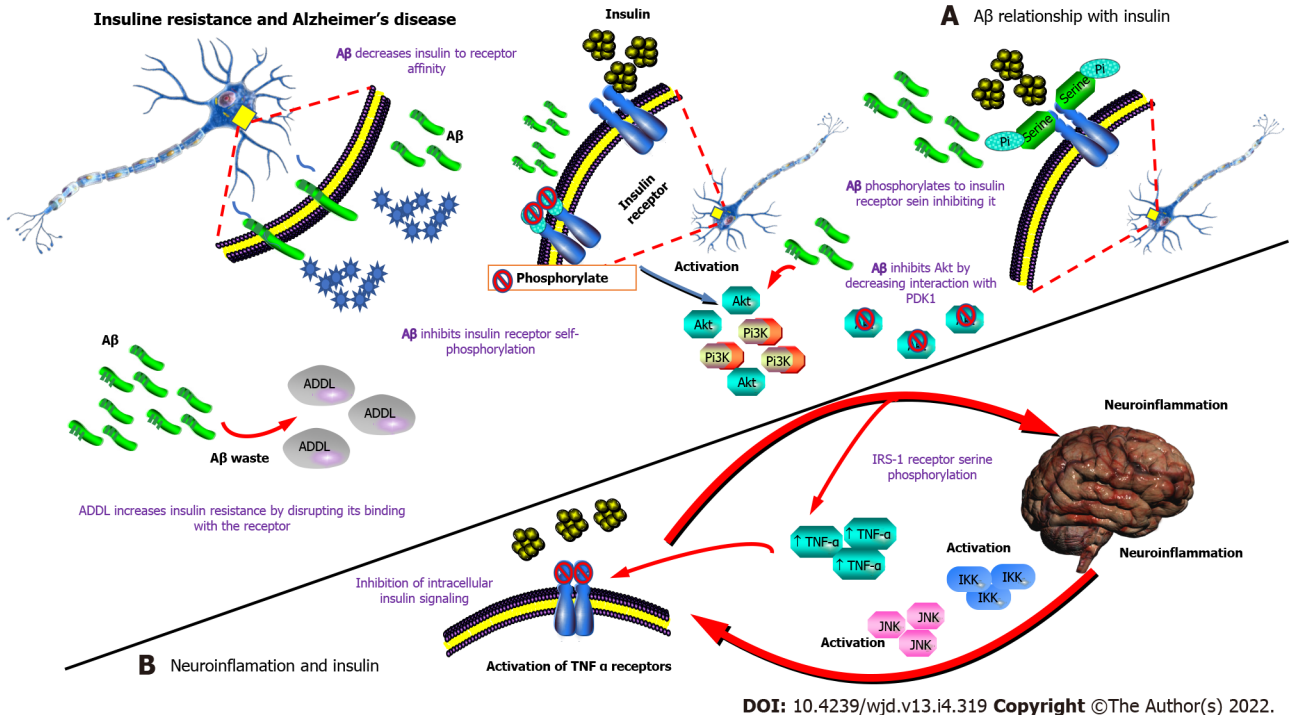

Figure 6 Insulin resistance and Alzheimer's disease. A: Amyloid beta (Aβ) relationship with insulin, the effect of Aβ plaques on insulin signaling, Aβ increases insulin resistance; B: Neuroinflammation and insulin inflammation disrupts insulin signaling pathways by impacting metabolic transducers. Some pictures were taken from Qiagen Pathways. Aβ: Amyloid beta; Pi3K: Phosphoinositide-3 kinase; Akt: Serine/threonine specific protein kinase; TNF-α: Tumor necrosis factor alpha.

the pathophysiology of DM-2 and AD[80] (Figure 7).

MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS IN AD

Metabolic dysfunction derived from oxidative stress and altered mitochondrial function has been extensively documented in AD. For example, increased levels of oxidative stress markers are correlated with the diminution in mitochondrial enzymatic activity in several brain areas and in peripheral tissues from AD patients[81,82]. The brain is very susceptible to oxidative stress due to its high content of polyunsaturated fatty acids, high oxygen comsumption, and low scavenging antioxidant capacity response system. Mitochondria are both generators and direct targets of reactive oxygen and reactive nitrogen species, thus oxidative stress and decreased mitochondrial activity of the oxidative phosphorylation system could have a significant effect on neuronal integrity.

Additionally, aging, a common risk factor for AD and insulin resistance, is also associated with decreased antioxidant capacity, increased oxidative stress, and decreased mitochondrial function.

The production of low levels of reactive oxygen species is not always detrimental: in peripheral tissues, the transient generation of reactive oxygen species facilitates insulin/IGF1 signaling by inhibiting phosphatases such as PTEN (which normally invert the PI3K signal)[82]. In the brain, the transient production of reactive oxygen species is used for synaptic transmission, facilitating long-term potentiation[82,83].

The first evidence for a link between insulin/IGF-1 signaling and mitochondrial dysfunction comes from experiments showing that insulin prevented the depolarization of the inner mitochondrial membrane in sensory neurons of diabetic rats[84]. Consistent with this, the activity of ATPase and the levels of the mitochondrial coenzyme Q9 were reduced in a model of DM. Thus, a decrease in the antioxidant activity of the mitochondria due to aging or alteration of insulin/IGF-1 signaling, in the case of metabolic disorders, could increase the vulnerability to AD since insulin prevents the decrease of oxidative phosphorylation and reduces oxidative stress induced by Aβ. Insulin stimulates mitochondrial protein synthesis, IGF-1 protects against hyperglycemia-induced oxidative stress, and the insulin/IGF-1 signaling defects makes neurons more vulnerable to reactive oxygen species[85]. Additionally, insulin regulates mitochondrial dynamics, autophagia, and tau phosphorylation in diabetic rats[86], and abolishes the Ab-40 mediated mitochondrial alterations[85], and increases mitochondrial ATP production in neuronal cells[87].

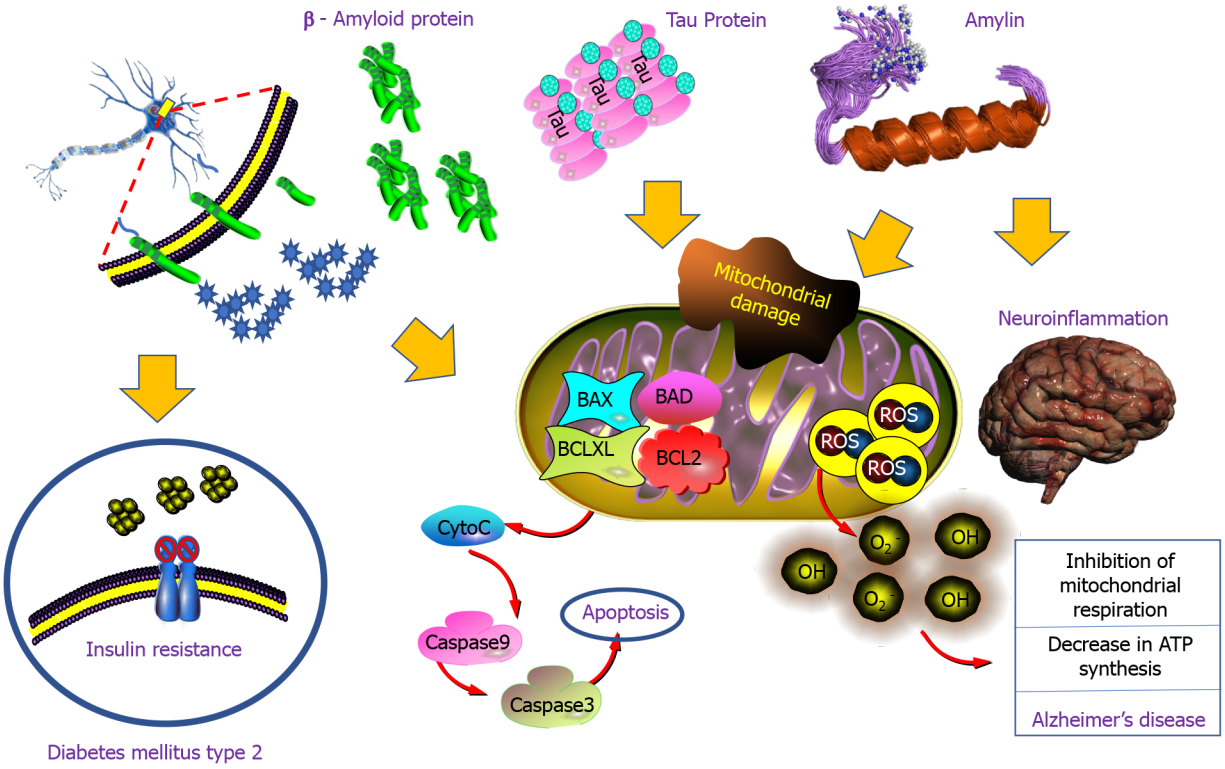

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Figure 7 Relation type 2 diabetes mellitus and Alzheimer's disease. The interconnection between β-amyloid protein, Tau protein and amylin increases the accumulation of amyloid deposit, oxidative stress, mitochondrial dysfunction, inflammation, insulin resistance and cell death; factors that explain the evolution to type 2 diabetes mellitus and Alzheimer's disease. Some pictures were taken from Qiagen Pathways.

Regardless of the exact sequence of events leading to neurodegeneration, there is strong evidence that mitochondrial dysfunction plays a key role in animal models of AD and DM. For example, the triple transgenic mouse model of AD (that expresses mutants of human APP, presenilin 1, and tau protein) presents both neuritic plaques and neurofibrillary tangles in brain. Furthermore, that triple transgenic model showed diminished mitochondrial respiratory activity and impaired oxidative phosphorylation systems. These alterations are correlated with the loss of synaptic integrity, with increased levels of reactive oxygen species, and with decreased levels of reduced glutathione and vitamin E. Interestingly, that alterations are comparable to those induced in wild-type mice treated with sucrose, is consistent with the proposal that mitochondrial alterations are associated with DM and that they contribute to the development of AD[88]. In accordance, NIRKO mice showed mitochondrial dysfunction, altered mitochondrial morphology, reduced expression of proteins of the oxidative phosphorylation system, and diminished mitochondrial respiratory activity(March 17,2022). Hence, alterations in the insulin/IGF-1 signaling, in DM for example, could lead to mitochondrial dysfunction, which would reduce the antioxidant capacity of the cell, and, when aggravated by aging, this would increase the vulnerability of the cell to Aβ-induced toxicity.

CONCLUSION

Finally, the appearance of cognitive impairement in DM-2 contributes to making the patient even more fragile and increasingly necessitating continuous home care to perform daily activities, which impacts the patients' quality of life. Interestingly, the insulin signaling pathway is hypothesized to be a specific target of therapeutic strategies to decrease alterations associated with age-related cognitive decline.

FOOTNOTES

Author contributions: Ortiz GG contributed coordination and work writing; Huerta M contributed diabetes expert and reviewer, and amyline contribution; González-Usigli HA and Mireles-Ramírez MA contributed neurologists and clinical work reviewers and writing; Torres-Sánchez ED and Delgado-Lara DL updated analysis of references, and graphic art; Pacheco-Moisés FP, Torres-Mendoza BM and Moreno-Cih RI contributed basic concepts, and their writing; Velázquez-Brizuela IE and Ortiz GG contributed neuropsychology and dementia concepts and writing.

Conflict-of-interest statement: This work does not present any conflict of interest.

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S-Editor: Gao CC
L-Editor: A
P-Editor: Wu RR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 338-357

DOI: 10.4239/wjd.v13.i4.338

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Roles of transient receptor potential channel 6 in glucose-induced cardiomyocyte injury

Shi-Jun Jiang

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Exbrayat JM, France; Song BW, South Korea; Waghmare S, India

Received: October 2, 2021
Peer-review started: October 2, 2021
First decision: January 12, 2022
Revised: January 18, 2022
Accepted: March 15, 2022
Article in press: March 15, 2022
Published online: April 15, 2022

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Abstract

BACKGROUND

Diabetic cardiomyopathy (DCM) is a serious complication of end-stage diabetes that presents symptoms such as cardiac hypertrophy and heart failure. The transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel that is closely related to the development of various cardiomyopathies.

AIM

To explore whether TRPC6 affects cardiomyocyte apoptosis and proliferation inhibition in DCM.

METHODS

We compared cardiac function and myocardial pathological changes in wild-type mice and mice injected with streptozotocin (STZ), in addition to comparing the expression of TRPC6 and P-calmodulin-dependent protein kinase II (P-CaMKII) in them. At the same time, we treated H9C2 cardiomyocytes with high glucose and then evaluated the effects of addition of SAR, a TRPC6 inhibitor, and KN-93, a CaMKII inhibitor, to such H9C2 cells in a high-glucose environment.

RESULTS

We found that STZ-treated mice had DCM, decreased cardiac function, necrotic cardiomyocytes, and limited proliferation. Western blot and immunofluorescence were used to detect the expression levels of various appropriate proteins in the myocardial tissue of mice and H9C2 cells. Compared to those in the control group, the expression levels of the apoptosis-related proteins cleaved caspase 3 and Bax were significantly higher in the experimental group, while the expression of the proliferation-related proteins proliferating cell nuclear antigen (PCNA) and CyclinD1 was significantly lower. *In vivo* and *in vitro*, the expression of TRPC6 and P-CaMKII increased in a high-glucose environment. However, addition of inhibitors to H9C2 cells in a high-glucose environment resulted in alleviation of

both apoptosis and proliferation inhibition.

CONCLUSION
The inhibition of apoptosis and proliferation of cardiomyocytes in a high-glucose environment may be closely related to activation of the TRPC6/P-CaMKII pathway.

Key Words: Diabetic cardiomyopathy; Apoptosis; Proliferation; H9C2 cells; Transient receptor potential channel 6; P-calmodulin dependent protein kinase II

Core Tip: Diabetic cardiomyopathy is a serious complication of end-stage diabetes that presents symptoms such as cardiac hypertrophy and heart failure. The transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel that is closely related to the development of various cardiomyopathies. We found that the inhibition of apoptosis and proliferation of cardiomyocytes in a high-glucose environment might be closely related to the activation of the TRPC6/P-calmodulin-dependent protein kinase II pathway.

Citation: Jiang SJ. Roles of transient receptor potential channel 6 in glucose-induced cardiomyocyte injury. *World J Diabetes* 2022; 13(4): 338-357
URL: https://www.wjgnet.com/1948-9358/full/v13/i4/338.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i4.338

INTRODUCTION

Diabetes is a disorder of glucose metabolism that is characterized by elevated blood glucose levels[1,2]. A serious complication of diabetes is diabetic cardiomyopathy (DCM), which can cause extensive necrosis of myocardial tissue, eventually resulting in heart failure and even cardiogenic shock[1,2]. The pathological manifestations of DCM are different from myocardial necrosis caused by ischemia and hypoxia of coronary heart disease or myocardial hypertrophy caused by simple hypertension. DCM is an independent and specific myocardial disease characterized by myocardial collagen precipitation and interstitial fibrosis. Cardiomyocyte apoptosis and a large amount of fibrous tissue proliferation are important manifestations of DCM[3-5], accompanied by over-activation of the renin angiotensin system (RAS)[6] and an increase in oxygen free radicals[7].

The RAS in the myocardium regulates cardiovascular function and promotes the growth of cardiomyocytes and vascular smooth muscles under normal conditions[8]. In a high-glucose (HG) environment, an increase in angiotensin II (AngII) in the RAS system results in an increase in the secretion of mineralocorticoid aldosterone, thereby causing an increase in the cardiac load[9]. Excessive secretion of cardiovascular endothelin can directly produce vasoconstrictors, induce vascular smooth muscle proliferation, and accelerate myocardial damage[10]. Simultaneously, endothelin stimulates the proliferation of myocardial fibroblasts and changes collagen metabolism, causing myocardial interstitial remodeling and affecting cardiac systolic and diastolic functions[11]. The increase in oxygen free radicals [reactive oxygen species (ROS)] is another important cause of myocardial damage in the HG environment. In diabetes, the level of lipid superoxide in muscle tissue increases significantly[12], while the expression of ROS-scavenging enzymes such as catalase and superoxide dismutase (SOD) in the myocardium is relatively low. Cardiomyocytes can easily become the target group of oxidative radicals and reactions. An *et al*[13] and Boyer *et al*[14] have shown that the use of antioxidants has a protective effect on myocardial cells in DCM, in addition to greatly improving the functional and morphological indexes of myocardial cells in DCM.

Canonical transient receptor potential channel 6 (TRPC6) is a non-selective cationic calcium channel protein with a molecular weight of 106 kDa[15]. As an important member of the transient receptor potential protein family, TRPC6 protein is expressed in various tissues such as the lung, heart, kidney, skin, and vascular system[16-19], and plays an important role in the physiological and pathophysiological processes of various cells. Apoptosis is a form of cell death and intracellular calcium overload is an important factor that triggers apoptosis[20]. Myocardial ischemia-reperfusion studies have found that selective knockout of TRPC6 can significantly reduce the degree of cardiomyocyte apoptosis and ischemia-reperfusion injury[21], suggesting that knockout of TRPC6 may inhibit ischemia-reperfusion-induced cardiomyocyte apoptosis, by inhibiting calcium influx. Calmodulin-dependent protein kinase II (CaMKII) is widely distributed in various tissues and cells, and is closely related to various life activities related to Ca^{2+}. In recent years, many studies have reported that CaMKII

plays an important role in various myocardial diseases, such as myocardial hypertrophy, myocardial infarction, and arrhythmia[22,23]. However, there are few reports on the interaction between TRPC6 and CaMKII, which warrants further research.

DCM pathogenesis is often related to the inflammatory process triggered by AngII, ROS, and extracellular regulated kinase (ERK)[24], suggesting that TRPC6 may be involved in DCM pathogenesis. Therefore, in the present study, a streptozotocin (STZ)-induced DCM mouse model was constructed to detect the expression of apoptosis-related proteins, including Bax, cleaved caspase 3 (CC3), and Bcl-2, and proliferation-related proteins, including proliferating cell nuclear antigen (PCNA) and CyclinD1. In addition, the model was used to evaluate the level of cardiomyocyte injury in a HG environment, to further explore the mechanism of apoptosis and proliferation inhibition induced by the HG environment through the TRPC6/P-CaMKII pathway *in vitro*. This study aimed to clarify the pathophysiological mechanism of DCM and provide a theoretical basis for identifying new targets for DCM treatment.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of Hubei Provincial Center for Disease Control and Prevention, and was conducted in accordance with the Declaration of Helsinki and the ARRIVE guidelines (https://arriveguidelines.org).

Reagents

Dimethyl sulfoxide (Sigma-Aldrich, United States), high-sugar Dulbecco's modified Eagle's medium (HyClone, United States), low-sugar Dulbecco's modified Eagle's medium (HyClone), Cell Counting Kit-8 (CCK-8; Beyotime, China), fetal bovine serum (Amresco, United States), radioimmunoprecipitation assay buffer (Amresco), protein concentration detection kit (Amresco), phenylmethyl sulfonyl fluoride (Amresco), cocktail protease inhibitor (Amresco), sodium dodecyl sulfate (SDS; Amresco), STZ (MedChemExpress, United States), KN-93 (MedChemExpress), Tween-20 (Amresco), SAR (Sigma-Aldrich), primary antibodies against TRPC6 (Abcam, United States), CC3 (Abcam), T-CAMKII (Abcam), P-CAMKII (Signalway Antibody, United States), Bcl-2 (Abcam), glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology, United States), Bax (Cell Signaling Technology), PCNA (Abcam), CyclinD1 (Cell Signaling Technology), and sheep anti-mouse secondary antibody (Abcam) were used in this study.

Experimental animals and protocol

Animal feeding: C57 mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. Twenty male C57 mice aged 6–8 wk, with good vital signs, were selected and raised in a standard animal room, with a relatively stable temperature and humidity.

Modeling method: STZ solution (at a concentration of 6.5 mg/mL) was prepared in citric acid buffer (pH 4.5). After 5 h of fasting, mice in the experimental group were intraperitoneally injected with STZ solution (15 μL/g) and those in the control group were injected with an equal amount of citric acid buffer for 3 d. After 7 d, the non-fasting blood glucose levels of the mice in each group were measured. When the non-fasting blood glucose level of the STZ mice exceeded 17 mmol/L, the model was considered to be successfully established.

Blood glucose measurement: One week and four weeks after the last administration, the non-fasting blood glucose levels of the mice were measured using peripheral blood. The tail tip was cut off with scissors, following which their peripheral blood was collected, and glucose levels in it were measured using a blood glucose meter (Abbott), when the mice were in a stress-free state.

The mice were then placed under deep anesthesia and euthanized by means of cervical dislocation, following which their myocardial tissue was collected for the experiment.

Cell culture

H9C2 cells were donated by the Laboratory of Anesthesiology Department of Union Hospital Affiliated with the Huazhong University of Science and Technology. After passage, the cells were divided into five groups according to the different culture media and drugs used: Low-glucose (LG) group–LG medium at a concentration of 5.5 mmoL/L; control group–275 μL of mannitol solution at a concentration of 500 mmoL/L was added to 5 mL of LG medium at a concentration of 5.5 mmoL/L, to obtain an osmotic pressure of 33 mmoL/L, as measured using OsmoPRO (Advanced Instruments Inc.); HG group–275 μL of glucose solution at a concentration of 500 mmoL/L was added to 5 mL of LG medium at a concentration of 5.5 mmoL/L, to obtain an osmotic pressure of 33 mmoL/L, as measured using OsmoPRO; high-glucose plus TRPC6 inhibitor group (HG + SAR)–sar7332 at a final concentration of 2 μmol/L was added to the HG medium at a concentration of 33.3 mmol/L; high-glucose plus CaMKII inhibitor group (HG + KN-93)–KN-93 at a final concentration of 5 μmol/L was added to the HG

medium at a concentration of 33.3 mmol/L. After 72 h, subsequent experiments were performed.

Western blot analysis

Proteins extracted from the mouse myocardium or H9C2 cells were separated by means of 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane with proteins was placed in skimmed milk powder for sealing. Primary antibody (1:500 dilution) was evenly dropped onto the PVDF membrane, which was then incubated in a shaker at 4 °C overnight. The primary antibody was eventually aspirated and the membrane was rinsed. Goat anti-rabbit IgG or goat anti-mouse IgG was diluted at a ratio of 1:1000 and added to the PVDF membrane box, which was shaken on a shaking table for 1 h. The chemiluminescent solution was mixed, configured, and poured onto the PVDF film with a pipette. After that, the strip drenched with the luminescent solution was placed into the developer for development. Finally, the strip was analyzed using the Image Lab (Bio-Rad Laboratories) and ImageJ (National Institutes of Health) software.

Immunofluorescence

Cell specimens and tissue sections were fixed with 4% paraformaldehyde for 15 min at room temperature. The samples were then washed with PBS (pH 7.4, 0.1 moL/L) three times, for 8 min each time. Triton X-100 was prepared at a concentration of 0.1% in PBS, and the samples were incubated with it at room temperature for 15 min. The samples were then washed with PBS three times, for 8 min each, and incubated with 3% donkey serum at room temperature for 25 min. The slides were washed again with warm PBS for 8 min. According to the manufacturer's instructions, the samples were incubated with primary antibodies against PCNA (at a dilution of 1:200), TRPC6 (1:400), and CC3 (1:400), following which the samples were placed in a refrigerator at 4 °C overnight. The slides were rinsed with PBS three times, for 8 min each. The samples were then incubated with anti-mouse or anti-rabbit secondary antibodies, used at a dilution ratio of 1:1000, in a 37 °C incubator for 2 h. The samples were washed three times with warm PBS, for 8 min each. An anti-fluorescence quenching sealing agent (ProLong Gold Antifade Reagent, Cell Signaling Technology) was added to the samples, the pictures of which were taken using a confocal microscope.

CCK-8 test

CCK-8 is a highly sensitive kit that is widely used to detect cell proliferation and toxicity. The cells were seeded at a density of 1000 cells/well into 96-well plates. Culture plates were pre-cultured at 37 °C in a humidified incubator containing 5% CO_2, following which 10 mL of CCK-8 was added to each well, and the 96-well plates were placed in an incubator for 1–4 h. The readings were taken by measuring the absorbance at a wavelength of 450 nm.

Lactate dehydrogenase detection

Lactate dehydrogenase (LDH) is an enzyme present in cells. When cells are stimulated or damaged, LDH is released from inside the cell to the outside. Thus, LDH levels are usually measured to evaluate the degree of apoptosis or necrosis. The principle of this experiment involves the conversion of lactic acid into pyruvate upon catalysis by LDH, accompanied by the reduction of NAD+ to nicotinamide adenine diphosphate hydride (NADH), which then reacts with 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolum chloride (INT) to produce trichromate methyl. This reaction leads to the generation of an absorption peak at a wavelength of 490 nm, which allows for the detection of LDH by means of colorimetry.

Cells in good condition were seeded into 96-well plates, at a density of 150 μL/well, and detected when the cell density reached 80%–85%. The old culture medium was then discarded, the cells rinsed with PBS, and a new culture medium added. After the pre-set time, the supernatant was aspirated and centrifuged for about 5 min at 300 *g*, following which the supernatant (120 μL) was added to the next culture plate and the readings were immediately determined.

ROS detection

ROS, including hydroxyl radicals, hydrogen peroxide, superoxide, and singlet oxygen, can damage nucleic acids and biofilms. Dichlorofluorescein diacetate (DCFH-DA) itself has no fluorescence, but can freely penetrate the cell membrane, to transform into DCFH inside the cells. ROS promotes the oxidation of DCFH to produce fluorescent substances. The levels of green fluorescence intensity were measured to estimate the level of intracellular ROS.

The cells were inoculated into 12-well plates with small slides, following which different drugs were added to the respective groups, and the cells were cultured for 72 h. When the cell density reached approximately 70%, DCFH-DA was directly added to the cells and they were incubated in the dark for 1 h, after reaching the initial concentration. The cells were then rinsed with PBS to remove residual DCFH-DA and reduce the background. The cells were observed and photographed under a fluorescence microscope and then collected and analyzed using flow cytometry.

Detection of apoptosis using flow cytometry

Annexin V, a phospholipid-binding protein, has high affinity for phosphatidylserine. It binds to the cell membranes of early apoptotic cells through the phosphatidylserine that is exposed outside these cells. Propidine iodide (PI) is a nucleic acid dye. However, in the middle and late stages of apoptosis and death, PI can penetrate the cell membrane and stain the nucleus red. Therefore, by matching the staining of annexin-V with PI, cells in the early and late stages of apoptosis can be detected.

The cells of each group were collected, re-suspended in the culture medium, and centrifuged at 4 °C for 5 min at 300 *g*, following which the supernatant was discarded. Pre-cooled PBS (1 mL) was then added to the cells, gently mixed, and then centrifuged at 400 *g* and 4 °C for 5 min. Post centrifugation, the supernatant was discarded, and the cells were re-suspended in 200 μL of PBS, to which 10 μL of fluorescein isothiocyanate (FITC)-annexin V and 10 μL of PI were added, gently mixed, and incubated at 4 °C in the dark for 30 min. PBS (300 μL) was then added to this system, followed by detection using flow cytometry. NovoExpress analysis software (Agilent) was used for the analysis.

Cell cycle detection using flow cytometry

The combination of PI and double-stranded DNA can produce fluorescence, the intensity of which is directly proportional to the double-stranded DNA content. After the DNA in the cell is stained with PI, the intracellular DNA content can be measured using flow cytometry, thereby helping in the analysis of the cell cycle.

We collected samples from each group. The collected cells (1×10^7) were re-suspended in 1 mL of medium and centrifuged at 400 *g* for 5 min, following which the supernatant was aspirated and discarded. The pellet was re-suspended in 300 μL of PBS, and 700 μL of anhydrous ethanol was added to it, following which this solution was placed in a refrigerator at -20 °C, to allow the cells to be fixed for more than 24 h. The fixed sample was then removed and centrifuged at 4 °C and 700 *g* for 5 min, following which the supernatant was aspirated and discarded. The cell pellet was washed twice with 1 mL of pre-cooled PBS, re-suspended in 0.5 mL of PI/RNase holding buffer, and incubated at room temperature for 15 min. The DNA content of the cells was measured using flow cytometry, to determine the proportion of cells in each cell cycle. The results were analyzed using NovoExpress software.

Histological analysis

Hematoxylin-eosin (HE) staining was used for the histological staining. Hematoxylin dye is alkaline, which mainly stains the chromatin in the nucleus and ribosome in the cytoplasm purple-blue. Eosin is an acidic dye that mainly stains components in the cytoplasm and extracellular matrix red. Masson's trichrome is one of the main methods used to stain fibers in tissues. It stains muscle fibers red and collagen fibers blue, and thus, helps distinguish collagen fibers from muscle fibers. Histologically, periodic acid–Schiff (PAS) staining is mainly used to detect glycogen or other polysaccharide substances; it stains glycogen and neutral mucilage material red and the nucleus blue.

Heart tissue was fixed with 10% formalin buffer, dehydrated with alcohol, and embedded in paraffin. We took 5 μm sections and stained them with HE, Masson's trichrome, and PAS. Photographs were taken using an optical microscope, while an Application Suite image system (Leica) was used to document the relevant parts of the sample.

Detection of myocardial injury markers

The main function of SOD is to catalyze the disproportionation of superoxide anion free radicals to hydrogen peroxide and oxygen. Superoxide anion free radicals are the normal metabolites in organisms. Creatine kinase (CK) is a dimer composed of two subunits, M and B. CK-MB in the myocardium accounts for approximately 10%–32%, so it is a marker of myocardial injury and has specificity. The apoptosis- or necrosis-mediated destruction of the cell membrane structure causes enzymes in the cytoplasm, such as LDH, to be released into the culture medium, with stable enzyme activity. Thus, we tested these three indicators using kits, according to the steps described in the manufacturer's instructions.

Echocardiographic detection

The mice were intraperitoneally injected with 3% sodium pentobarbital (40 mg/kg). After reaching a state of mild anesthesia, they were fixed on the animal fixation plate in the supine position. The mice were then touched with fingers, for apical pulsation. The hair in the heart region was removed and an appropriate amount of coupling agent was applied to the heart. The anterior zone was measured using an echocardiogram (Philips). A 2–4 MHz ultrasound probe was placed on the left side of the sternum, to show the short-axis section of the left ventricle.

Statistical analysis

All data were analyzed using Prism 6.0 (GraphPad). For the measurement data, the variance homogeneity test was carried out first, followed by the unpaired two-tailed *t*, *t'*, or rank-sum test, as needed. Statistical significance was set at $P < 0.05$.

RESULTS

A flow chart of the animal and cell experiments is shown in Supplementary Figure 1.

Establishment of a mouse model of DCM

After STZ was injected into the mice of the experimental group, the blood glucose levels of the mice in each group were measured regularly (Table 1). The fasting blood glucose levels of mice in the control group were in the normal range, whereas after 8 wk of STZ injection, the fasting blood glucose levels of mice in the model group increased significantly compared to those before the injection ($P < 0.001$) and those in the control group ($P < 0.001$), which confirmed the successful establishment of the diabetes mouse model. Long-axis ultrasound imaging and cardiac function measurement of the left ventricle showed that compared to those in the control group, there was a significant decrease ($P < 0.05$) in the left ventricular end diastolic diameter, left ventricular ejection fraction, and left ventricular fraction shortening in the STZ group, while the left ventricular end systolic diameter increased significantly ($P < 0.05$), which suggested decreased cardiac function (Figure 1A and Table 2). Upon detection of myocardial injury markers, as compared to those in the control group, the levels of LDH and CK-MB increased significantly ($P < 0.0001$) in the STZ group, while SOD decreased significantly ($P < 0.0001$), accompanied by the generation of a large amount of ROS ($P < 0.0001$), indicating that there was serious oxidative stress injury in the myocardial tissue of mice in the STZ group (Figure 1B and Table 3). HE staining showed that compared to those in the control group, the cells in the STZ group were disordered and hypertrophic, with obviously broken and dissolved myocardial fibers. Masson's staining showed that cardiomyocytes in the STZ group were hypertrophic and necrotic, and obvious fibrous tissue hyperplasia appeared in the myocardial stroma. PAS staining showed that glycogen vacuoles, mucus, and myocardial interstitial inflammatory cell infiltration increased in the STZ group (Figure 1C). The above results indicated that compared to that in the normal control group, the myocardial tissue in the STZ group had obvious pathological damage, which further confirmed that the DCM model was established successfully.

Apoptosis of cardiomyocytes in DCM mice

Cardiomyocyte apoptosis plays an important role in DCM pathogenesis. To explore the apoptosis of cardiomyocytes in the HG environment, we performed the following experiments: (1) The myocardial tissue of the two groups was made into cell suspensions and flow cytometry was performed on them, which showed that there was a significant increase ($P < 0.0001$) in the apoptotic rate of cardiomyocytes in the DCM model group (Figure 2A); (2) Western blot was used to detect the expression of apoptosis-related proteins in the myocardium, which was compared to that in the control group, and it was found that there was no significant difference in the level of cardiomyocyte apoptosis-related protein Bcl-2 in the DCM model group ($P > 0.05$), while the expression of Bax and CC3 increased significantly, and there was obvious apoptosis ($P < 0.01$) (Figure 2B); and (3) Immunofluorescence showed that there was a significant enhancement ($P < 0.01$) in the fluorescence intensity of CC3 in the DCM model group (Figure 2C).

Proliferation of cardiomyocytes in DCM mice

After the onset of DCM, calcium overload in cardiomyocytes and excessive energy consumption by mitochondria might lead to a decrease in the number of cells. In order to explore the proliferation of cardiomyocytes in the HG environment, we carried out the following experiments: (1) The results of flow cytometry showed that the ratio of myocardial cells in the G1 phase in the DCM model group increased significantly ($P < 0.01$), indicating that the proliferation of many cells in the G1 phase was blocked (Figure 3A); (2) The cell cycle-related proteins in the two groups were detected using Western blot, which showed that compared to those in the control group, the expression levels of PCNA and CyclinD1 decreased significantly in the DCM model group ($P < 0.01$) (Figure 3B); and (3) Immunofluorescence also showed that there was a significant decrease ($P < 0.01$) in the expression of PCNA in the myocardial tissue of mice in the model group (Figure 3C). These results suggested that cardiomyocyte proliferation was significantly inhibited.

Expression levels of TRPC6 and P-CAMKII proteins in the myocardium of DCM mice

To clarify the role of TRPC6 in high glucose-induced cardiomyocyte injury, we detected the expression of TRPC6 and its related P-CaMKII signaling pathway in DCM mice. The myocardial tissues in the control and DCM model groups were analyzed using Western blot. Compared to those in the control group, the expression levels of calcium channel proteins, such as TRPC6 and P-CAMKII, increased significantly ($P < 0.01$) in the DCM model group (Figure 4A). The myocardial tissues of the two groups were analyzed using immunofluorescence, the results of which showed that there was a significant increase ($P < 0.001$) in the expression of TRPC6 in the myocardium of the model group (Figure 4B).

Table 1 Blood glucose levels in control and streptozotocin injection groups (mmo/L)

Group	*n*	0 wk	1 wk	4 wk	8 wk	12 wk
Control	10	6.3 ± 0.6	6.8 ± 0.6	6.7 ± 0.5	6.5 ± 0.3	6.4 ± 0.9
STZ	10	6.1 ± 0.5	16.1 ± 8.2	14.6 ± 3.8	19.2 ± 5.7[a,b]	23.4 ± 4.3[a,b]

[a]$P < 0.001$, compared with that before modeling.
[b]$P < 0.001$, compared with the control group.
STZ: Streptozotocin.

Table 2 Comparison of cardiac ultrasound results between control and streptozotocin injection groups in left ventricular end diastolic diameter, left ventricular end systolic diameter, left ventricular ejection fraction, and fraction shortening

Group	*n*	LVEDD (mm)	LVESD (mm)	LVEF (%)	LVFS (%)
Control	10	2.9 ± 0.4	0.95 ± 0.3	82 ± 2.1	67 ± 2.9
STZ	10	3.6 ± 0.3[a]	0.81 ± 0.5[a]	69 ± 3.2[b]	48 ± 1.8[c]

Compared with the control group:
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
STZ: Streptozotocin; LVEDD: Left ventricular end diastolic diameter; LVESD: Left ventricular end systolic diameter; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fraction shortening.

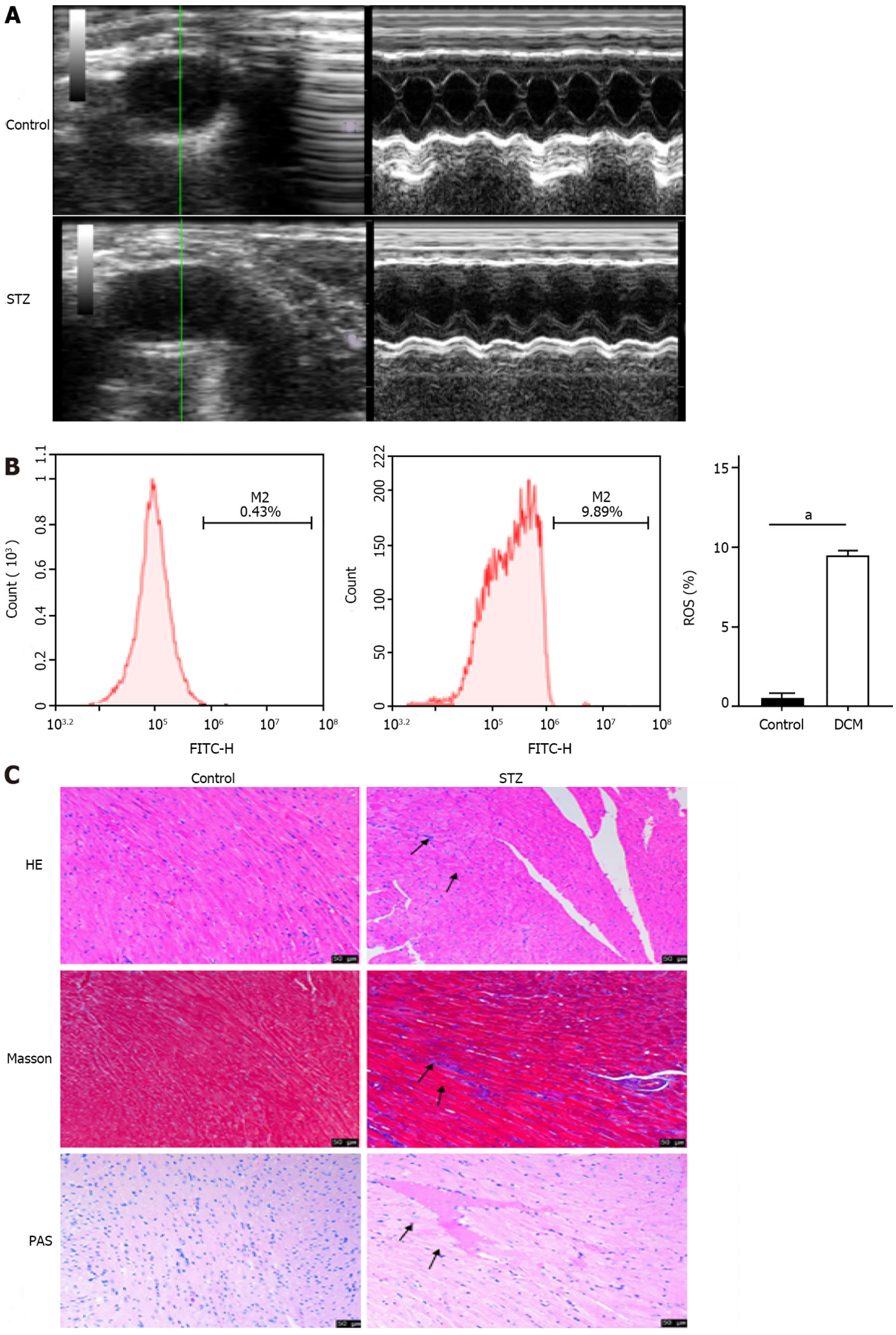
Table 3 Detection of myocardial injury markers in control and streptozotocin injection groups

Group	*n*	LDH (mU/mg)	CK-MB (U/g)	SOD (U/mg)
Control	10	424.7 ± 5.2	11.3 ± 0.5	67.5 ± 2.3
STZ	10	1027.6 ± 4.3[a]	48.2 ± 0.6[a]	19.8 ± 1.2[a]

Compared with the control group:
[a]$P < 0.0001$.
STZ: Streptozotocin; LDH: Lactate dehydrogenase; SOD: Superoxide dismutase.

Effects of SAR7334 and KN-93 on the pathological and biochemical changes of HG-induced H9C2 cardiomyocytes

To clarify the role and mechanism of TRPC6 and CaMKII in DCM-induced cardiomyocyte injury, H9C2 cardiomyocytes were treated with HG, to simulate DCM-induced cardiomyocyte injury *in vitro*. At the same time, the cells were pre-treated with SAR7334, a TRPC6 inhibitor, and KN-93, a CaMKII inhibitor, to evaluate their roles in HG-induced cardiomyocyte injury. Compared to the LG and control groups, the HG group displayed obvious cell necrosis and floating, with a significant decrease ($P < 0.05$) in the number of cells. In addition, compared to the HG group, there was alleviation of cell necrosis and a significant increase ($P < 0.05$) in the number of cells in the SAR7334 and KN-93 treatment groups, which suggested that the inhibition of TRPC6 and CaMKII might lessen HG-induced cardiomyocyte injury. The absorbance of each group at the wavelength of 450 nm was detected using the CCK-8 method, to evaluate the H9C2 cell proliferation in each group. The results showed that compared to the LG and control groups, there was a significant decrease ($P < 0.01$) in the viability of cells in the HG group (33 mmol/L), while the viabilities of cells in the HG + SAR and HG + KN-93 groups were significantly higher than those in the HG group ($P < 0.05$) (Figure 5A). LDH is widely distributed in the myocardium and brain, and participates in redox reactions in the cytoplasm. The amount of LDH released and mortality of cardiomyocytes are commonly used to evaluate the degree of cardiomyocyte injury. The OD value is an index that reflects LDH activity. Upon examination of LDH activity and cardiomyocyte mortality, compared to those in the control and LG groups, the OD value of cells increased significantly in the HG group, while the HG-induced LDH release was significantly inhibited in the HG + SAR and HG + KN-93 groups. Compared to those of the control and LG groups, the cell mortality of the HG group increased significantly, while compared to that of the HG group, the cell mortalities of the HG + SAR and HG + KN-93 groups decreased significantly (Figure 5B). Excessive ROS can cause serious



DOI: 10.4239/wjd.v13.i4.338

Figure 1 Long axis ultrasound imaging and cardiac function measurement of the left ventricle. A: Left ventricular short axis view and cardiac

function were measured in the control group and streptozotocin (STZ) injection group; B: Flow cytometry of reactive oxygen species in the control group and STZ injection group; C: HE, Masson, and periodic acid Schiff staining of diabetic cardiomyopathy and control mice. Scale bars = 50 μm. $^{a}P < 0.0001$. STZ: Streptozotocin; ROS: Reactive oxygen species; PAS: Periodic acid Schiff.

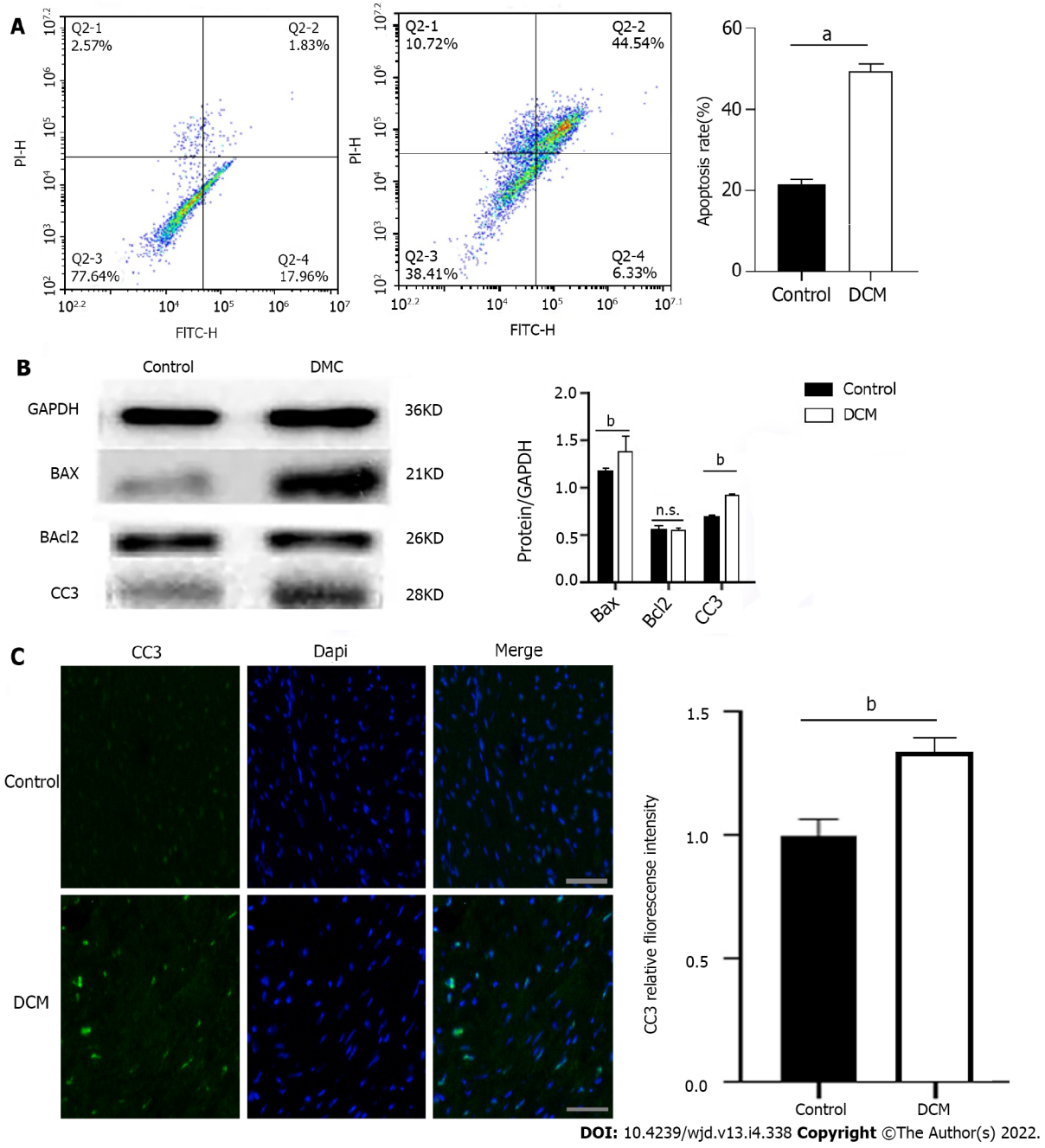


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Figure 2 Apoptosis of cardiomyocytes in diabetic cardiomyopathy mice. A: Apoptosis of diabetic cardiomyopathy (DCM) and control mice was detected using double staining of FITC-Annexin V and propidine iodide and the representative images of flow cytometry are presented. $^{a}P < 0.0001$; B: Expression levels of apoptosis related proteins BAX, cleaved Caspase 3 (CC3), and Bcl2 in DCM mice and control mice. $n = 5$, unpaired t-test, $^{b}P < 0.01$. n.s.: No statistical difference; C: Fluorescence intensity of CC3 in cardiac myocytes of DCM and control mice. Scale bars = 20 μm, $^{b}P < 0.01$. DCM: Diabetic cardiomyopathy.

damage to cytoplasmic proteins and nucleic acids, and is closely related to apoptosis. Upon detection of ROS fluorescence using DCFH-DA, the fluorescence intensity of the HG group was found to be significantly higher than those of the LG and control groups ($P < 0.0001$), while the levels of ROS in the cardiomyocytes of the HG + SAR and HG + KN-93 groups decreased significantly ($P < 0.0001$) (Figure 5C).

Effects of SAR7334 and KN-93 on HG-induced apoptosis of H9C2 cells

In order to further clarify whether the decline of cardiomyocyte viability induced by HG is related to

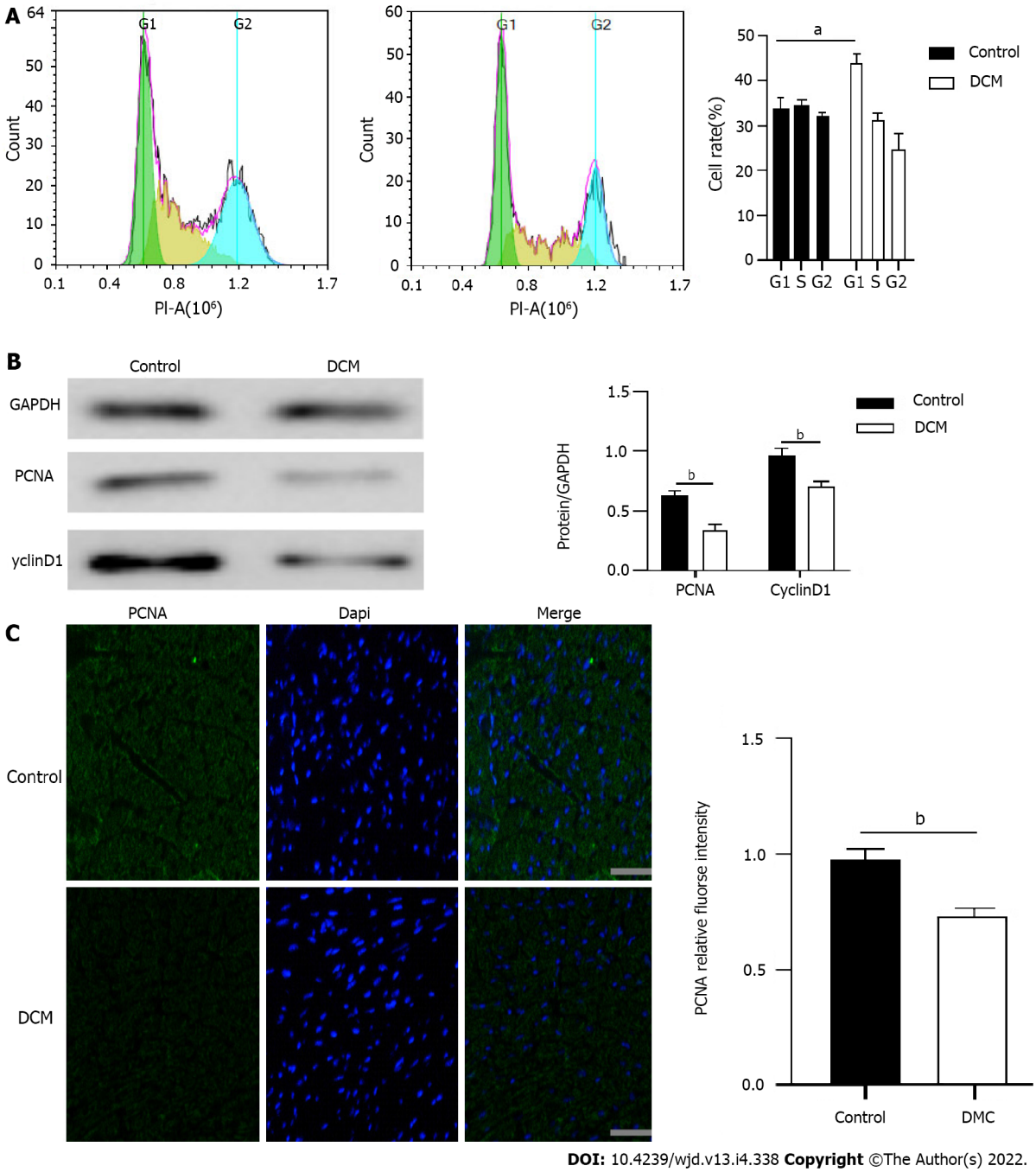

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Figure 3 Proliferation of cardiomyocytes in diabetic cardiomyopathy mice. A: Cell cycle was detected by flow cytometry for the percentage of cells in each cell cycle of diabetic cardiomyopathy (DCM) and control mice. n = 5, unpaired t-test, $^{a}P < 0.001$; B: The expression levels of cardiac cell cycle related proteins PCNA and CyclinD1 in DCM and control mice. n = 5, unpaired t-test, $^{b}P < 0.01$; C: Fluorescence intensity of PCNA in cardiac myocytes of DCM and control mice. Scale bars = 20 μm, $^{b}P < 0.01$. DCM: Diabetic cardiomyopathy.

mediation of apoptosis, we induced the cells using HG (33 mmoL/L) for 72 h and then detected the apoptosis of cells in each group using flow cytometry. Compared to those in the LG and control groups, the apoptosis rate was significantly higher ($P < 0.0001$) in the HG group; however, upon treatment with SAR (2 μmoL/L) and KN-93 (5 μmoL/L), as compared to that in the HG group, the apoptosis rates in the HG + SAR and HG + KN-93 groups decreased significantly ($P < 0.0001$) (Figure 6A). Moreover, when Western blot was used to detect the changes in the levels of apoptosis-related proteins including Bax, CC3, and Bcl-2 upon induction with HG, the expression levels of Bax and CC3 were found to be significantly up-regulated ($P < 0.001$), indicating that the H9C2 cells showed obvious apoptosis. However, compared to those in the HG group, the levels of Bax and CC3 proteins decreased significantly in the HG + SAR and HG + KN-93 groups ($P < 0.001$) (Figure 6B). Immunofluorescence results showed that the fluorescence intensity of CC3 increased significantly in the HG group ($P < 0.001$), while it decreased significantly ($P < 0.01$) in the HG + SAR and HG + KN-93 groups (Figure 6C and D). These results suggested that the effects of SAR7334 and KN-93 are similar, and that they both

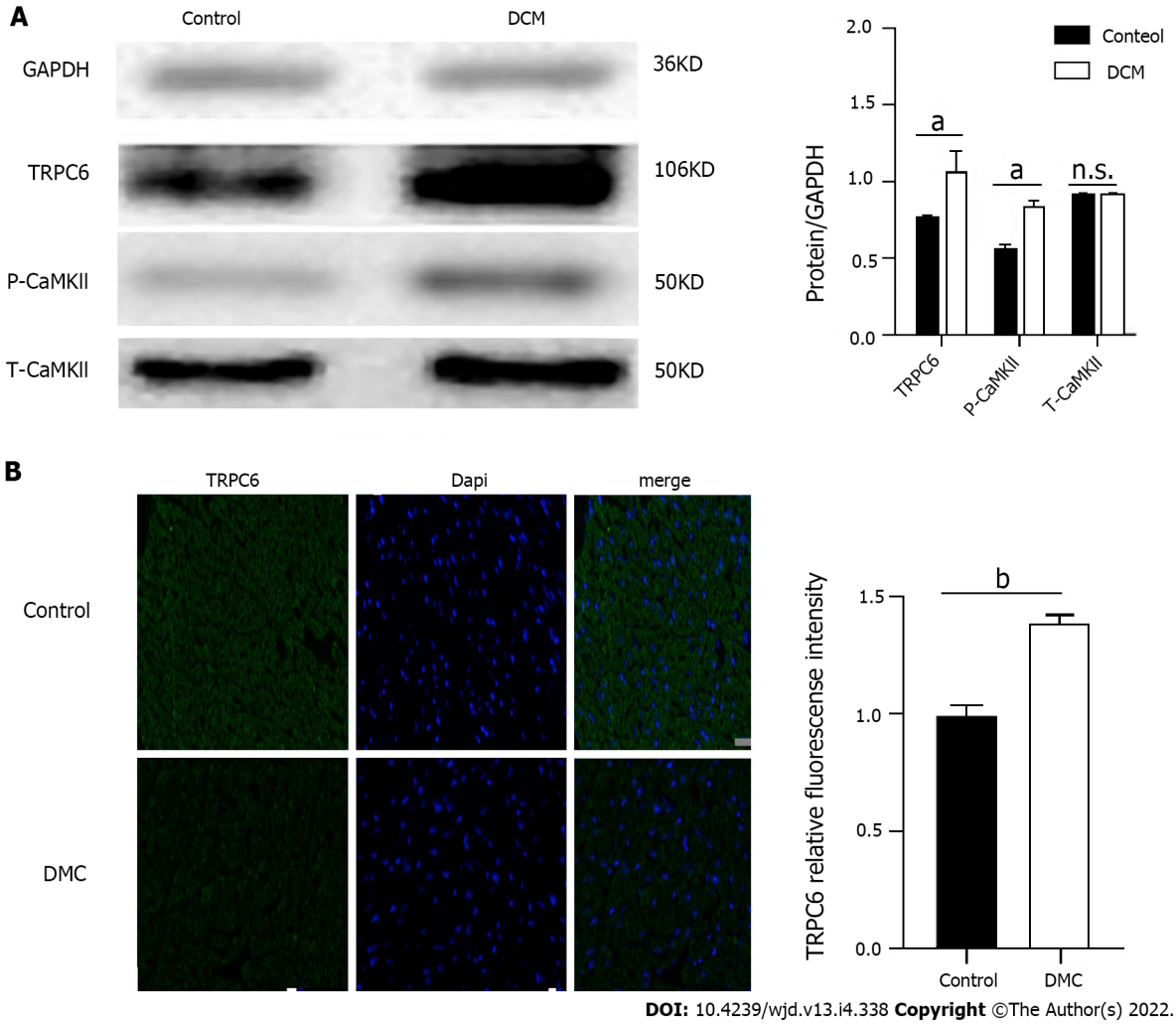

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Figure 4 Expression of transient receptor potential channel 6 and P-CAMKII proteins in the myocardium of diabetic cardiomyopathy mice. A: Expression levels of calcium channel protein transient receptor potential channel 6 (TRPC6) and P-CAMKII in cardiac myocytes of diabetic cardiomyopathy (DCM) and control mice. $n = 5$, unpaired *t*-test, $^{a}P < 0.01$. n.s.: No statistical difference; B: Fluorescence intensity of TRPC6 in cardiac myocytes of DCM and control mice. Scale bars = 20 μm, $^{b}P < 0.001$. DCM: Diabetic cardiomyopathy; TRPC6: Transient receptor potential channel 6.

inhibit HG-induced apoptosis and protect cardiomyocytes.

Effects of SAR7334 and KN-93 on HG-induced inhibition of H9C2 cell proliferation

In order to further explore whether calcium overload can inhibit the proliferation of cardiomyocytes, we detected the cell cycle in different treatment groups using flow cytometry. Compared to those in the LG and control groups, the ratio of cells in G1 phase was significantly higher ($P < 0.001$) in the HG group, while as compared to that in the HG group, the ratio of cells in G1 phase was significantly lower ($P < 0.01$) in the HG + SAR7334 and HG + KN-93 groups (Figure 7A). In addition, when the expression levels of PCNA and CyclinD1 were detected using Western blot, as compared to those in the LG and control groups, the expression levels of PCNA and CyclinD1 were significantly down-regulated ($P < 0.0001$) in the HG group, while as compared to those in the HG group, the expression levels of PCNA and CyclinD1 were significantly up-regulated in the HG + SAR7334 and HG + KN-93 groups ($P < 0.001$) (Figure 7B). The results of immunofluorescence showed that as compared to those in the LG and control groups, there was a significant decrease ($P < 0.0001$) in the PCNA fluorescence intensity in the HG group, while as compared to that in the HG group, groups that were treated with SAR7334 and KN-93 displayed a significant increase ($P < 0.01$) in PCNA fluorescence intensity (Figure 7C and D). These results showed that calcium overload inhibited cell proliferation, but the cell proliferation improved upon addition of SAR7334 and KN-93.

Changes in TRPC6 and P-CAMKII expression upon intervention with SAR7334 and KN-93

In order to explore the specific mechanism of cardiomyocyte injury caused by HG, Western blot was used to detect the expression of TRPC6 and P-CAMKII in the different treatment groups. As compared to those in the LG and control groups, there was a significant increase ($P < 0.001$) in the expression

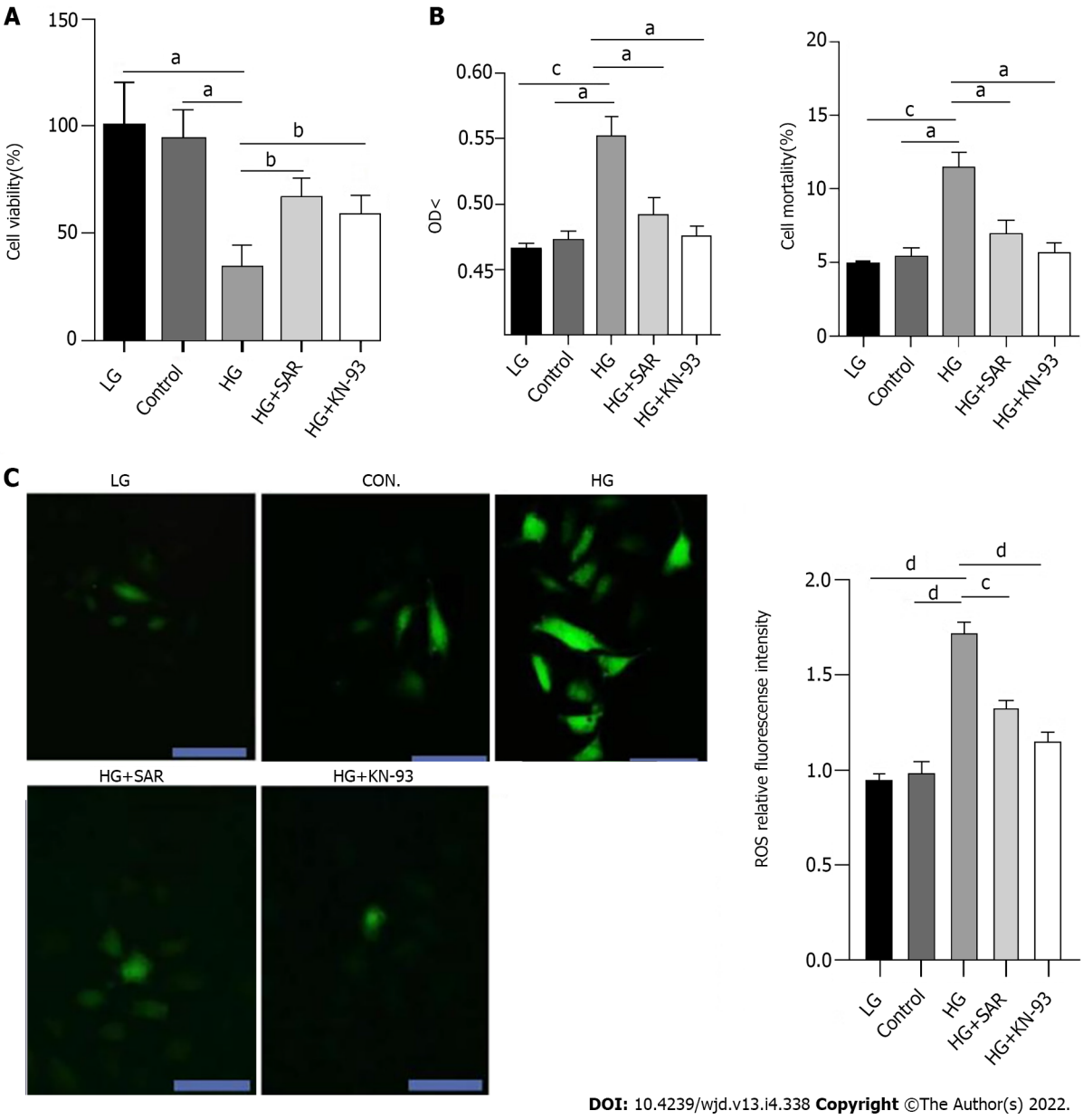

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Figure 5 Pathological and biochemical changes of H9C2 cardiomyocytes in each group. A: After high glucose induced injury of H9C2 cells, the effects of SAR7334 and KN-93 on the proliferation of H9C2 cells were observed by Cell Counting Kit-8 method. $^{a}P < 0.01$, $^{b}P < 0.05$; B: After high glucose induced injury of H9C2 cells, lactate dehydrogenase method was used to detect the effects of SAR and KN-93 on the mortality of H9C2 cells. $n = 5$, unpaired t-test, $^{a}P < 0.01$, $^{c}P < 0.001$; C: Reactive oxygen species fluorescence intensity of each group after treatment with fluorescent probe DCFH-DA. $n = 5$, unpaired t-test, scale bars = 20 μm, $^{c}P < 0.001$, $^{d}P < 0.0001$. HG: High-glucose; LG: Low-glucose.

levels of TRPC6 and P-CAMKII in the HG group, while compared to those in the HG group, there was a significant decrease ($P < 0.001$) in the expression levels of TRPC6 and P-CAMKII in the HG + SAR and HG + KN-93 groups (Figure 8A). Immunofluorescence also showed that, as compared to those in the LG and control groups, there was a significant increase ($P < 0.0001$) in the fluorescence intensity of TRPC6 in the HG group, while the same decreased significantly ($P < 0.0001$) upon addition of SAR7334 and KN-93, suggesting that HG might activate the TRPC6/P-CAMKII pathway, cause intracellular calcium overload, and finally result in cardiomyocyte injury (Figure 8B and C).

DISCUSSION

Normal metabolic function is important for maintaining homeostasis of the internal environment and normal functioning of various organs. Metabolic dysfunction can cause pathophysiological changes, from the level of the tissue cells to that of the whole body, through various mechanisms, such as imbalance of internal environment homeostasis, activation of inflammatory pathways, and activation of

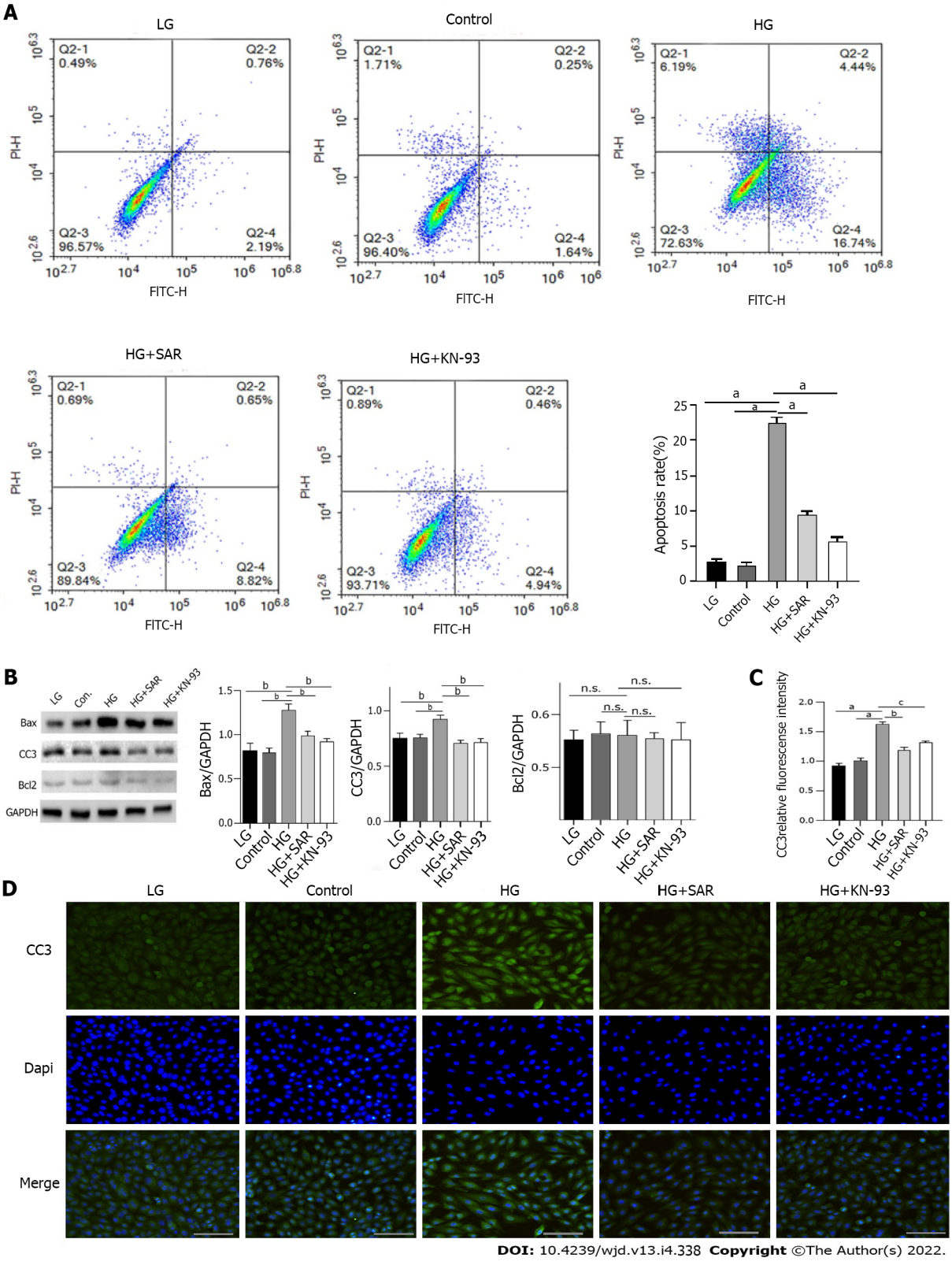

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Figure 6 Effects of SAR7334 and KN-93 on high-glucose-induced apoptosis of H9C2 cells. A: Apoptosis rate of H9C2 cells in each group was detected by flow cytometry. n = 5, unpaired t-test, $^{a}P < 0.0001$; B: Effects of SAR7334 and KN-93 on the expression of apoptosis related proteins Bax, cleaved Caspase 3 (CC3), and BCl2 in H9C2 cells induced by high glucose. n = 5, unpaired t-test, $^{b}P < 0.001$. n.s.: No statistical difference; C and D: Expression of apoptosis protein CC3 detected by immunofluorescence. n = 5, unpaired t-test, $^{a}P < 0.0001$, $^{b}P < 0.001$, $^{c}P < 0.01$. Scale bars = 10 μm. HG: High-glucose; LG: Low-glucose.

apoptosis-related pathways[25-27]. Diabetes is caused by congenital genetic or acquired pathogenic factors. It manifests as a series of metabolic syndromes that are characterized by abnormal blood glucose elevation[28,29]. Diabetes usually leads to the activation of signaling pathways such as HIPPO/YAP [30], NF-κB/NLRP3[31], and Erk/Nrf2/HO-1[32], which leads to pathological changes in peripheral blood vessels, smooth muscles, and cardiomyocytes, manifested as diabetic foot, diabetic nephropathy,

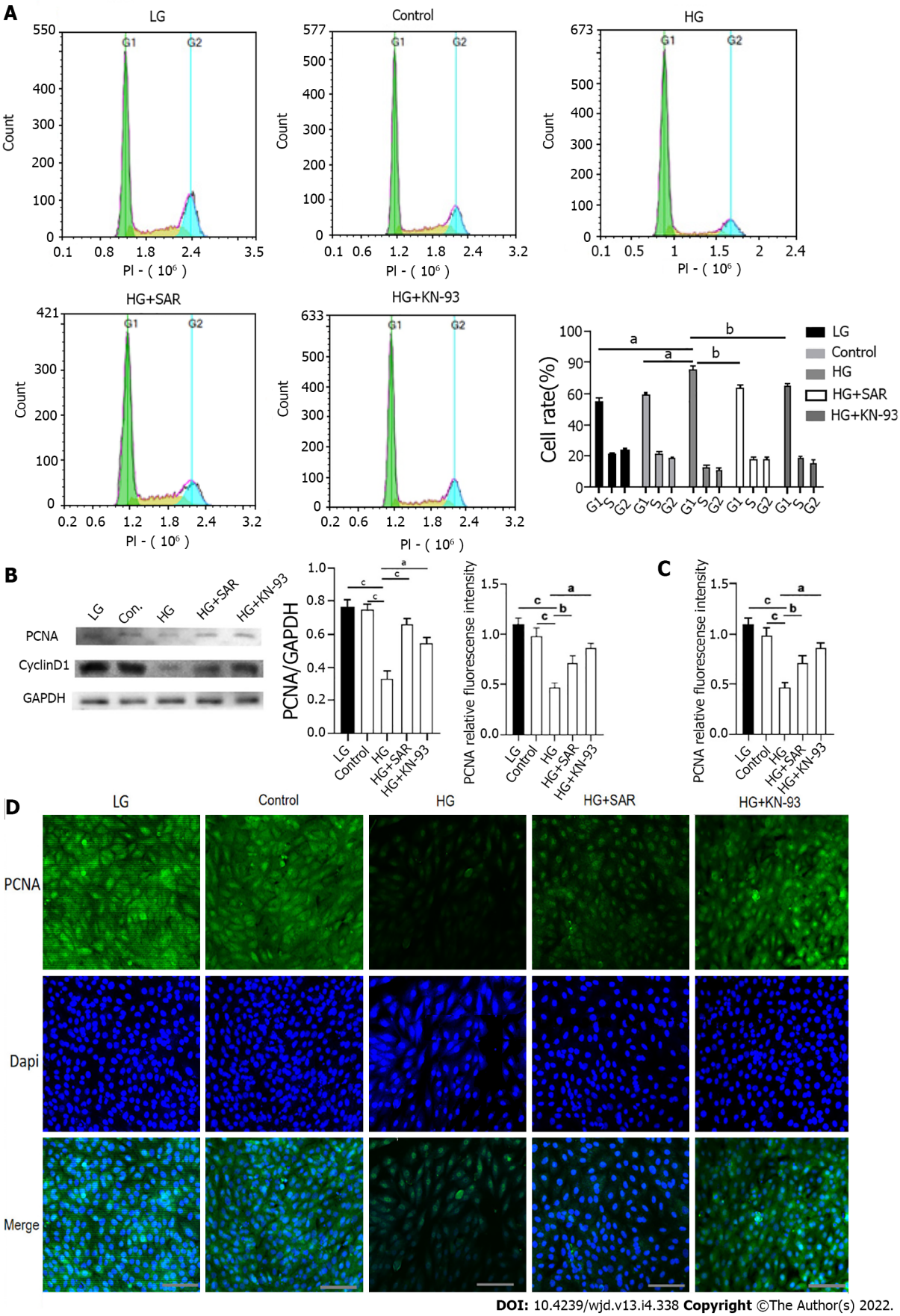

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Figure 7 Effects of SAR7334 and KN-93 on high-glucose-induced inhibition of H9C2 cell proliferation. A: Percentage of cells in each group in each period was detected by flow cytometry. $n = 5$, unpaired t-test, $^{a}P < 0.001$, $^{b}P < 0.01$; B: Effects of SAR7334 and KN-93 on the expression levels of cycle related proteins PCNA and CyclinD1 in H9C2 cells induced by high glucose. $n = 5$, unpaired t-test, $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.0001$. n.s.: No statistical difference; C and D:

Expression of PCNA detected by immunofluorescence. n = 5, unpaired t-test, $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.0001$. Scale bars = 10 μm. HG: High-glucose; LG: Low-glucose.

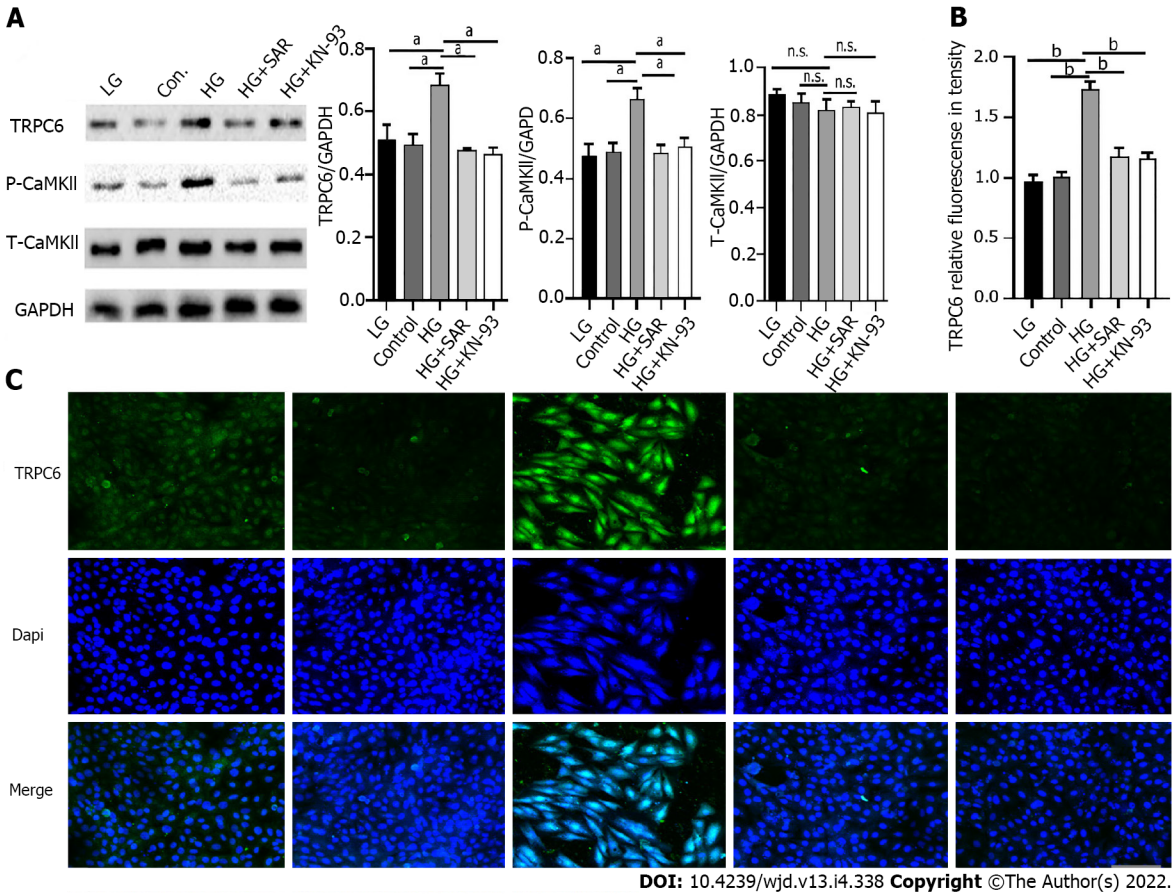

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Figure 8 Changes of expression of transient receptor potential channel 6 (TRPC6) and P-CaMKII under the intervention of SAR7334 and KN-93. A: Effects of SAR7334 and KN-93 on the expression of transient receptor potential channel 6 (TRPC6) and P-CaMKII in high glucose induced H9C2 cells. n = 5, unpaired t-test, $^{a}P < 0.001$. n.s.: No statistical difference; B and C: Expression of TRPC6 protein detected by immunofluorescence. n = 5, unpaired t-test, $^{b}P < 0.0001$. Scale bars = 10 μm. HG: High-glucose; LG: Low-glucose; TRPC6: Transient receptor potential channel 6.

DCM, and other complications. Such complications cause an increase in the mortality rate of the patients [33-36]. DCM is a serious complication of diabetes[37] that is characterized by irreversible damage to myocardial structure and function in diabetic patients, due to hyperglycemia and metabolic disorders [38]. In terms of pathogenesis, studies have shown that DCM is an independent and specific myocardial injury, and the pathogenesis of DCM is different from that of hypertensive heart disease, coronary atherosclerotic heart disease, and other heart diseases[39,40]. Our present study suggests that HG levels might activate inflammatory pathways, to induce apoptosis and fibrous tissue proliferation in diabetic patients, thus playing an important role[41,42].

TRPC6 is a non-selective cation channel protein. It has been reported that TRPC6 can regulate Ca^{2+} influx under physiological conditions. *In vitro*, Sonneveld *et al*[43] found that the HG environment caused up-regulation of TRPC6 expression in an AngII expression-dependent manner[43]. Zhang *et al* [44] found that AngII could up-regulate the expression of TRPC6 in podocytes, increase Ca^{2+} influx, and promote podocyte apoptosis and autophagy[44]. Studies have shown that when the expression of TRPC6 increases, the intracellular Ca^{2+} concentration increases, resulting in the activation of calmodulin (CaM) and translocation of nuclear factor for activated T cells 2 (NFAT2) in the cytoplasm to the nucleus, which binds to the corresponding homeopathic elements in the target gene promoter, to trigger the expression of downstream molecules[45]. Elucidating the exact mechanism of TRPC6 in DCM pathogenesis has important theoretical and practical significance for the development of relevant therapeutic targets. This study aimed to explore the role of TRPC6 in the pathogenesis of DCM, using STZ to construct the DCM model and simulate the HG environment of cells *in vitro*.

In the present study, a DCM model was constructed by means of intraperitoneal injection of STZ. To clarify whether STZ can induce DCM effectively, we used HE, Masson's trichrome, and PAS pathological staining to detect changes in myocardial structure after modeling. Our results showed that

compared to the control group, there was an increase in cardiomyocyte hypertrophy/necrosis, collagen fiber proliferation, and glycogen vacuoles in the STZ group. This suggests that STZ could effectively induce DCM. To clarify the mechanism of STZ-induced DCM-related phenomena and to determine if it involved cardiomyocyte apoptosis, we performed flow cytometry, Western blot, and immunofluorescence, and found that in the STZ-injected group, there was a significant increase in the apoptosis rate, in addition to a significant increase in the expression of Bax and CC3 as well as fluorescence intensity of CC3, suggesting that the HG environment might aggravate the degree of cardiomyocyte apoptosis. The same experimental method was also used to detect the cell cycle, the results for which showed that in the model group, the ratio of cardiomyocytes in the G1 phase significantly increased, while the expression of PCNA and CyclinD1 as well as the PCNA fluorescence intensity of cardiomyocytes decreased obviously, which suggested that HG might inhibit the proliferation of cardiomyocytes. The expression levels of TRPC6 and P-CaMKII were also detected and found to be significantly higher in the model animals, as compared to those in the controls, suggesting that HG might promote cardiomyocyte apoptosis by activating the expression of TRPC6 and P-CaMKII.

Pathological oxidative stress and metabolic dysfunction can mediate cardiomyocyte injury through activation of the RAS and ROS systems[46]. Studies have shown that the activation of the ROS signaling pathway is an important mechanism of cell injury[46,47]. It has been reported that the production of intracellular ROS can directly damage the mitochondrial membrane and cause mutations in mitochondrial DNA, resulting in an imbalance between oxidative stress and Ca^{2+} regulation[47]. At the same time, ROS can also activate nuclear factor-kappaB (NF-κB) indirectly, such as by activating caspase-3 and its activator, thereby inducing the high expression of apoptosis-related genes[48]. As a non-selective calcium channel, TRPC6 can induce cell injury through calcium overload[49]. To further clarify the specific roles of TRPC6 in DCM, we examined the effect of TRPC6 on cardiomyocytes in the HG environment using *in vitro* cell culture experiments.

Under a light microscope, we found that there was severe apoptosis of H9C2 cells in a HG environment. SAR7334 and KN-93 are specific inhibitors of TRPC6 and CaMKII, respectively. Upon addition of SAR7334 and KN-93, there was alleviation of cell injury. The CCK-8 assay showed that SAR7334 and KN-93 had a protective effect on cardiomyocytes. LDH is widely distributed in the myocardium and brain, and participates in redox reactions in the cytoplasm. The amount of LDH released and mortality of cardiomyocytes are commonly used to evaluate the degree of cardiomyocyte injury. By detecting the LDH level in the supernatant of cultured cells, we confirmed that inhibition of CaM could alleviate the toxic effects of HG on cardiomyocytes. DCFH-DA was used to detect ROS. It was found that under HG stimulation, compared to that of the HG group, the content of ROS in cardiomyocytes of the HG + SAR and HG + KN-93 groups decreased significantly, indicating that the production of ROS is very important in the process of HG-induced apoptosis, which is regulated by the concentration of Ca^{2+}. The activation of TRPC6 induced by HG further caused intracellular calcium overload and increased the expression of CaM, which was the initiating factor leading to apoptosis; in addition, the ROS downstream further activated the apoptosis pathway.

To clarify whether HG can induce the apoptosis and proliferation inhibition of cardiomyocytes and elucidate the related pathways, we used flow cytometry, Western blot, and immunofluorescence to detect the rate of apoptosis, cell cycle, and expression of apoptosis-/cell cycle-related proteins. In the HG group, the apoptosis rate increased significantly, in addition to which, a greater number of cells were also blocked in the G1 phase. After application of SAR and KN-93, there was a decrease in the apoptosis rate, as well as the proportion of cells that were blocked in the G1 phase. The expression of apoptosis-related proteins, including Bax and CC3, increased significantly in the HG group, with the expression of TRPC6 and P-CaMKII showing the same trend. The expression of cell cycle-related proteins, including PCNA and cyclinD1, decreased significantly in the HG group, but improved upon addition of calcium channel inhibitors. These results suggested that the change in Ca^{2+} concentration might play an important role in this situation.

CONCLUSION

In conclusion, the results of the flow cytometry, immunofluorescence, and Western blot experiments suggested that the HG environment might lead to cardiomyocyte apoptosis and inhibition of cardiomyocyte proliferation, by inducing the TRPC6/P-CaMKII pathway. The *in vitro* experiments further validated that inhibition of TRPC6 and CaMKII might protect cells in the HG environment, improve cell activity, reduce cell mortality, and promote cell proliferation.

ARTICLE HIGHLIGHTS

Research background

Diabetic cardiomyopathy (DCM) is a serious complication of end-stage diabetes that presents symptoms

such as cardiac hypertrophy and heart failure. The transient receptor potential channel 6 (TRPC6) protein is a very important selective calcium channel that is closely related to the development of various cardiomyopathies.

Research motivation

In recent years, many studies have reported that calmodulin-dependent protein kinase II (CaMKII) plays an important role in various myocardial diseases, such as myocardial hypertrophy, myocardial infarction, and arrhythmia. However, there are few reports on the interaction between TRPC6 and CaMKII, which warrants further research.

Research objectives

The purpose of this study was to explore whether TRPC6 affects cardiomyocyte apoptosis and proliferation inhibition in DCM.

Research methods

We compared cardiac function and myocardial pathological changes in wild-type mice and mice injected with streptozotocin (STZ), in addition to comparing the expression of TRPC6 and P-calmodulin-dependent protein kinase II (P-CaMKII) in them. At the same time, we treated H9C2 cardiomyocytes with high glucose and then evaluated the effects of addition of SAR, a TRPC6 inhibitor, and KN-93, a CaMKII inhibitor, to such H9C2 cells in a high-glucose environment.

Research results

We found that STZ-treated mice had DCM, decreased cardiac function, necrotic cardiomyocytes, and limited proliferation. Western blot and immunofluorescence were used to detect the expression levels of various appropriate proteins in the myocardial tissue of mice and H9C2 cells. Compared to those in the control group, the expression levels of the apoptosis-related proteins cleaved caspase 3 and Bax were significantly higher in the experimental group, while the expression of the proliferation-related proteins proliferating cell nuclear antigen and CyclinD1 were significantly lower. *In vivo* and *in vitro*, the expression of TRPC6 and P-CaMKII increased in a high-glucose environment. However, addition of inhibitors to H9C2 cells in a high-glucose environment resulted in alleviation of both apoptosis and proliferation inhibition.

Research conclusions

The inhibition of apoptosis and proliferation of cardiomyocytes in a high-glucose environment may be closely related to activation of the TRPC6/P-CaMKII pathway.

Research perspectives

This study might provide a new insight for the treatment of DCM.

FOOTNOTES

Author contributions: Jiang SJ conceived and designed the study, collected, analyzed, and interpreted the data, wrote the manuscript, provided critical revisions that are important for the intellectual content, and approved the final version of the manuscript.

Institutional review board statement: This study was conducted with approval from the Ethics Committee of Hubei Provincial Center for Disease Control and Prevention (No. 202110009).

Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals.

Conflict-of-interest statement: There are no relevant financial or non-financial interests to disclose.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Cai YX

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 358-375

DOI: 10.4239/wjd.v13.i4.358

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Long noncoding RNA X-inactive specific transcript regulates NLR family pyrin domain containing 3/caspase-1-mediated pyroptosis in diabetic nephropathy

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Specialty type: Urology and nephrology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ma D, China; Zhang L, China

Received: October 15, 2021
Peer-review started: October 15, 2021
First decision: December 27, 2021
Revised: January 24, 2022
Accepted: March 15, 2022
Article in press: March 15, 2022
Published online: April 15, 2022

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Abstract

BACKGROUND

NLRP3-mediated pyroptosis is recognized as an essential modulator of renal disease pathology. Long noncoding RNAs (lncRNAs) are active participators of diabetic nephropathy (DN). X inactive specific transcript (XIST) expression has been reported to be elevated in the serum of DN patients.

AIM

To evaluate the mechanism of lncRNA XIST in renal tubular epithelial cell (RTEC) pyroptosis in DN.

METHODS

A DN rat model was established through streptozotocin injection, and XIST was knocked down by tail vein injection of the lentivirus LV sh-XIST. Renal metabolic and biochemical indices were detected, and pathological changes in the renal tissue were assessed. The expression of indicators related to inflammation and pyroptosis was also detected. High glucose (HG) was used to treat HK2 cells, and cell viability and lactate dehydrogenase (LDH) activity were detected after silencing XIST. The subcellular localization and downstream mechanism of XIST were investigated. Finally, a rescue experiment was carried out to verify that XIST regulates NLR family pyrin domain containing 3 (NLRP3)/caspase-1-mediated RTEC pyroptosis through the microRNA-15-5p (miR-15b-5p)/Toll-like receptor 4 (TLR4) axis.

RESULTS

XIST was highly expressed in the DN models. XIST silencing improved renal metabolism and biochemical indices and mitigated renal injury. The expression of inflammation and pyroptosis indicators was significantly increased in DN rats

and HG-treated HK2 cells; cell viability was decreased and LDH activity was increased after HG treatment. Silencing XIST inhibited RTEC pyroptosis by inhibiting NLRP3/caspase-1. Mechanistically, XIST sponged miR-15b-5p to regulate TLR4. Silencing XIST inhibited TLR4 by promoting miR-15b-5p. miR-15b-5p inhibition or TLR4 overexpression averted the inhibitory effect of silencing XIST on HG-induced RTEC pyroptosis.

CONCLUSION

Silencing XIST inhibits TLR4 by upregulating miR-15b-5p and ultimately inhibits renal injury in DN by inhibiting NLRP3/caspase-1-mediated RTEC pyroptosis.

Key Words: Diabetic nephropathy; Pyroptosis; Renal tubular epithelial cell; Long noncoding RNA X-inactive specific transcript; microRNA-15b-5p; Toll-like receptor 4; NLR family pyrin domain containing 3/caspase-1 pathway

Core Tip: We investigated the mechanism of long noncoding RNA X-inactive specific transcript (XIST) on NLR family pyrin domain containing 3/caspase-1-mediated renal tubular epithelial pyroptosis through the microRNA-15-5p/Toll-like receptor 4 axis and identified XIST as a possible molecular target to mediate renal tubular epithelial pyroptosis in the treatment of renal injury in diabetic nephropathy.

Citation: Xu J, Wang Q, Song YF, Xu XH, Zhu H, Chen PD, Ren YP. Long noncoding RNA X-inactive specific transcript regulates NLR family pyrin domain containing 3/caspase-1-mediated pyroptosis in diabetic nephropathy. *World J Diabetes* 2022; 13(4): 358-375

URL: https://www.wjgnet.com/1948-9358/full/v13/i4/358.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i4.358

INTRODUCTION

As a deleterious complication of diabetes and a critical cause of end-stage renal failure, diabetic nephropathy (DN) mainly manifests as morphological and functional abnormalities, such as glomerular hyperfiltration, natriuresis, proteinuria, the dysregulation of cell junction intercellular communication [1] and changes in the structure and function of the kidneys[2]. DN is characterized by thickening of the glomerular basement membranes, glomerular capillary damage, inflammation and oxidative stress, mesangium expansion, and urinary microalbumin[3]. The main process crucial for DN pathogenesis is the increased loss of renal cells, which consequentially leads to renal damage and renal dysfunctions involving alterations in structural integrity and glomerular filtration[4]. DN is triggered by many factors, such as dyslipidaemia, hyperglycaemia, haemodynamic abnormalities, and environmental and genetic causes[5,6]. Additionally, evidence accumulated from experimental and clinical studies indicates that renal inflammation is key to determining the progression of renal injury[7]. Despite the high mortality of diabetic patients with DN, effective treatment remains elusive. Identifying genetic determinants and understanding their roles in DN progression is crucial to develop effective diagnostic tools and treatments.

Pyroptosis, a newly discovered mode of programmed cell death, is characterized by dependence on caspase-1 and NLRP3 inflammasome activation[8]. The participation of pyroptosis in cancers, cardiovascular diseases, and microbial infection-related diseases has been intensively studied[9-11]. Pyroptosis can cause cell death, inflammation, and renal injury, while inhibiting pyroptosis relieves pathological injury[12,13]. The involvement and importance of pyroptosis in DN have recently been clarified[14]. Increasing evidence suggests that pyroptosis and the subsequent inflammatory response play key roles in DN pathogenesis[15-17].

Noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs, participate in DN pathogenesis by regulating inflammation, apoptosis, and other pathological processes[18,19]. A recent study reported that DN progression is closely related to lncRNA expression, which is important for the early diagnosis of DN and targeted interventions[20]. XIST expression is elevated in the serum of DN patients[21]. Silencing XIST protects against renal interstitial fibrosis in DN mice *via* miR-93-5p[22]. XIST also regulates pyroptosis in lung cancer[23], but its effect on renal tubular epithelial cell (RTEC) pyroptosis is unknown. miRNAs are well-known biomarkers used in DN diagnosis, and miRNA profile alterations significantly correlate with DN and renal dysfunction [24]. miR-15b-5p is downregulated in urinary exosomes of DN patients compared with healthy controls [6]. Moreover, the miR-15a-5p-XIST-CUL3 competing endogenous RNA (ceRNA) network is involved in

the mechanism of sepsis-induced acute kidney injury[25]. Whether there is a ceRNA loop involving XIST and miR-15b-5p in RTECs in DN progression is largely unknown. Therefore, we studied the mechanism of lncRNA XIST in RTEC pyroptosis and provide a new theoretical basis for XIST as a molecular target to mediate RTEC pyroptosis in DN management.

MATERIALS AND METHODS

DN model establishment and treatment

Male Sprague–Dawley rats (8 wk, 300-320 g) were provided by Shanghai Experimental Animal Center (Shanghai, China) and raised in a specific pathogen-free animal room at 25 °C under a 12/12 h light-dark cycle with free access to food and water. The model was established after one week of adaptive feeding. Streptozotocin (STZ, 50 mg/kg, S0130, Sigma, United States) was used to establish the DN rat model. STZ was dissolved in 0.1 M citric acid buffer. The DN model was established by injecting STZ (intraperitoneal injection, i.p.) once per day for 5 d. Fasting blood glucose levels were measured 5-7 d after injection, and rats with fasting blood glucose levels above 16.5 mmol/L for 3 consecutive days were identified as successful DN models. Rats in the control group were injected with an equal volume of citrate buffer (P4809, Sigma, United States). The rats were allocated into a control group, DN group, DN + LV-sh-NC group, and DN + LV-sh-XIST group. Each rat was numbered according to its weight and divided into groups according to the random number method. There were 8 rats in each group, for a total of 32 rats. The health status of the rats was monitored every 2 d. If weight loss was > 15%, the rat was euthanized; however, no animals died before the end of the experiment. After successful model establishment (Day 7 after STZ injection), the DN rats were injected with LV-sh-NC or LV-sh-XIST lentiviral interference vectors (200 μL, GenePharma, Shanghai, China). All DN rats were sacrificed by intraperitoneal injection of pentobarbital (100 mg/kg, P-010, Sigma United States) on the 21st day after STZ injection. All appropriate measures were taken to minimize the pain and discomfort of the animals.

Detection of renal metabolic and biochemical indices in rats

The rats were placed in the metabolic cage alone, and urine protein for 24 h (UP 24 h) was collected. On the 20th day after STZ injection, rats in each group fasted overnight and then weighed. Tail vein blood samples were then analyzed by a portable blood glucose meter (OneTouch Ultra Easy, Johnson & Johnson, NJ, United States) to measure fasting blood glucose (FBG). Blood samples were collected from the posterior orbital venous plexus. Serum samples were separated from the blood samples by centrifugation (1000 g, 10 min, 4 °C). The rats were euthanized by excessive pentobarbital (100 mg/kg, i.p.), and the kidney was collected and weighed. KW/BW was calculated according to the following formula:

KW/BW = kidney weight (g)/body weight (g).

According to the manufacturer's instructions, blood urea nitrogen (BUN) was detected by a UREA/BUN assay kit (Changchun Huili Biotech, Changchun, China); serum creatinine (Cr) was detected by a Cr assay kit (Huili Biotech); and UP 24 h was detected by an UP assay kit (Nanjing Jiancheng, Nanjing, China). These parameters were analyzed with a 240 or 800 Automated Chemistry Analyser (Rayto, Shenzhen, China).

Histological staining of kidney tissue

The rats were sacrificed, and the kidneys were collected. The kidneys were fixed with 16 g/L paraformaldehyde solution for 24 h and then embedded in paraffin. Serial sections of 4 μm thickness were cut from paraffined blocks and subjected to haematoxylin and eosin (HE), periodic acid Schiff (PAS), and Masson's trichrome staining and observed under a light microscope (TI-S, Nikon, Japan). The pathological analysis was performed by 2 pathologists unaware of this study using ImageJ (NIH, Bethesda, MD, United States). The glomerular volume was calculated as follows: $V = 4/3\ \pi\ (D/2)^3$, where D is the geometric mean of the four diameters. The mesangial matrix index was calculated as follows: mesangial matrix index = mesangial matrix area/global area × 100%. To evaluate tubulointerstitial fibrosis, at least 20 visual fields of each section were analyzed by Masson staining software. Scores from 0 to 3 were used to assess the degree of tubulointerstitial fibrosis: 0, normal interstitium; 0.5, ≤ 5% of area injured; 1, 5%-15% of area injured; 1.5, 16%-25% of area injured; 2, 26%-35% of area injured; 2.5, 36%-45% of area injured; 3, > 45% of area injured[26].

Enzyme-linked immunosorbent assay

Fresh kidney tissue was homogenized in phosphate-buffered saline (PBS) and centrifuged. After total protein quantification, renal tissue samples were used for enzyme-linked immunosorbent assay (ELISA) measurement[27]. At the same time, cell culture supernatant was collected. The expression levels of interleukin-1β (IL-1β, RLB00) and IL-18 (DY521-05) in renal tissue and cells were determined according to the ELISA kit instructions (R&D Systems, Minneapolis, MN, United States).

Western blot analysis

The tissue or cells were lysed with enhanced RIPA lysate (AR0105, Boster, Wuhan, Hubei, China) containing protease inhibitor, and the protein concentration was assessed using a bicinchoninic acid protein quantitative kit (Boster). The protein samples were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% bovine serum albumin (A1933, Sigma, United States) for 2 h to block nonspecific binding. Next, the membranes were immersed overnight in primary antibody solutions, followed by secondary goat anti-rabbit IgG H&L (HRP) (1:2000, ab205718, Abcam, Cambridge, United Kingdom) or goat anti-mouse IgG (1:2000, ab205719, Abcam, Cambridge, United Kingdom). The proteins were developed using enhanced chemiluminescence (EMD Millipore, Billerica, MA, United States) and analysed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, United States). The primary antibodies were cleaved caspase-1 (1:1000, #89332, CST, Danvers, MA, United States), caspase-1 (1:100, sc-392736, Santa Cruz Biotechnology, Dallas, TX, United States), rabbit monoclonal antibody gasdermin D (GSDMD, 1:1000, ab219800, Abcam, Cambridge, United Kingdom), GSDMD-N (1:1000, PA5-116815, Thermo Fisher, Waltham, MA, United States), NLRP3 (1:1000, ab214185, Abcam, Cambridge, United Kingdom), ASC mouse monoclonal antibody (1:100, sc-514414, Santa Cruz Biotechnology, TX, United States), TLR4 rabbit polyclonal antibody (1:500, ab13867, Abcam, Cambridge, United Kingdom), and GAPDH (1:2500, ab9485, Abcam, Cambridge, United Kingdom).

Immunofluorescence

The kidney was placed on a clean workbench, and the fibrous capsule was stripped using an aseptic technique. Next, 1 cm^3 of renal tubular epithelial tissue was removed from the outer skin tissue, washed with normal saline, and fixed with 10% formalin. Sections 4 μm thick were incubated overnight with caspase-1 mouse monoclonal antibody (1:50, sc-392736, Santa Cruz Biotechnology) at 4 °C, and then conjugated with secondary goat anti-mouse IgG (Alexa Fluor® 488) (1:200, ab150113, Abcam, Cambridge, United Kingdom) in the dark for 1 h. After washing in PBS, the slides were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (D9542, Sigma, United States) for 3 min, followed by glycerine loading. The fluorescence was evaluated under a fluorescence microscope (Olympus Life Sciences, Tokyo, Japan).

Cell culture and transfection

The human tubular endothelial cell line HK2 from ATCC (Rockville, MD, United States) was cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA, United States) containing 10% (v/v) foetal bovine serum (FBS, F2442; Sigma, St. Louis, Missouri, United States) at 37 °C with 50 mL/L CO_2. After 24 h of starvation in serum-free medium, HK2 cells were cultured in normal (5 mmol/L, NG) or high (40 mmol/L, HG) glucose (Y0001745, Sigma, United States) for 48 h[28].

The miR-15b-5p mimic and miR-15b-5p inhibitor, siRNA sequence targeting XIST (si-XIST), overexpression TLR4 plasmid (oe-TLR4), and their corresponding controls were provided by GenePharma (Shanghai, China). The cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States).

The si-XIST sequences were as follows: si-XIST-1 (SS sequence: GGAAGUACCUACUACUUAAGA, AS Sequence: UUAAGUAGUAGGUACUUCCAG), si-XIST-2 (SS sequence: GGUGGACUAUCAACAUAUAAU, AS sequence: UAUAUGUUGAUAGUCCACCAG), and si-XIST-3 (SS sequence: GGAAAUAGAUAAAUGUCAAAG, AS sequence: UUGACAUUUAUCUAUUUCCUU).

Quantitative real-time polymerase chain reaction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, United States). A reverse transcription kit (RR047A, Takara, Tokyo, Japan) was used to obtain cDNA. A miRNA first-strand cDNA synthesis kit (B532451-0020, Sangon, Shanghai, China) was used for miRNA detection.

SYBR Premix Ex Taq II (DRR081, Takara) and real-time fluorescence qPCR (7500, ABI, Foster City, CA, United States) were used for the qRT–PCR experiment. The PCR primers (Table 1) were designed and synthesized by Sangon. GAPDH or U6 was the internal reference. Gene expression was calculated using the $2^{-\Delta\Delta t}$ method.

Cell viability measurement

The viability of HK2 cells was detected by a Cell Titer Glo Luminescent Cell Viability Assay Kit (G7570, Promega, Beijing, China). Cells (10^5) were seeded into 6-well plates containing normal growth medium and then collected when the confluence reached 80%-90%. Before collecting the HK2 cells, the reagents were prepared, and then the cells (50 μL) were loaded into black-walled 96-well plates that contained the same amount of final substrate solution. After mixing for 10 min, the cells were incubated in a plate-reading luminometer (Varioskan™, Thermo Scientific, Rockford, IL, United States).

Lactate dehydrogenase (LDH) release assay

An LDH release assay kit (Beijing, Nanjing, China) was used to detect cytotoxicity. HG-treated HK2 cells were plated into 96-well plates and incubated at 37 °C with 50 mL/L CO_2 for 24 h. The cell medium was

Table 1 Primer sequences

	Forward Primer (5'-3')	Reverse Primer (5'-3')
XIST (human)	AGCTCCTCGGACAGCTCTAA	CTCCAGATAGCTGGCAACC
XIST (Norway rat)	CCCATACCCTCATACCCTAATG	GGCTGGCCTCATTTCTGGGCTC
hsa-miR-15b-5p	ATCCAGTGCGTGTCGTG	TGCTTAGCAGCACATCATG
rno-miR-15b-5p	GCCGAGTAGCAGCACATCATG	CTCAACTGGTGTCGTGGA
hsa-pre-miR-15b	GGCCTTAAAGTACTGTAGCAGC	CTCAACTGGTGTCGTGGA
rno-pre-miR-15b	GGAGTTTTTCCCTTTTGGATG	ATAATGATTCGCATCTTGATTGTAG
TLR4 (human)	CCGCTCTGGCATCATCTTCA	TGGGTTTTAGGCGCAGAGTT
TLR4 (Norway rat)	GCCAGGATGATGCCTCTCTTGC	CCTCAGGTCAAAGTTGTTGC
U6 (human)	GCTCGCTTCGGCAGCACA	GAGGTATTCGCACCAGAGGA
U6 (Norway rat)	ATGGCGGACGACGTAGATCAGCA	TCAGCCAACTCTCAATGGAGGGG
GAPDH (human)	TCCATGACAACTTTGGCATC	CATGTCAGATCCACCACGGA
GAPDH (Norway rat)	CAAGATGGTGAAGGTCGGTGT	CTTACTCCTTGGAGGCCATGTAG

XIST: X inactive specific transcript; TLR4: toll like receptor 4; hsa: *Homo sapiens*; rno: *Rattus norvegicus*; U6: U6 small nuclear RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; pre: precursor; miR: microRNA.

then collected, and LDH activity was measured.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was used to identify the subcellular localization of XIST in HK2 cells. In accordance with the instructions of the Ribo™ lncRNA FISH probe mix (Red) (RiboBio, Guangzhou, Guangdong, China), the cover glass was placed into the 6-well culture plate, and HK2 cells were seeded onto it. The confluence was approximately 80% after 1 d of culture. The slides were then washed with PBS and fixed with 1 mL of 16 g/L paraformaldehyde. The slides were treated with proteinase K (2 μg/mL), glycine, and ethylphthalein reagent and then incubated with 250 μL of prehybridizing solution at 42 °C for 1 h. Next, the prehybridizing solution was removed, and 250 μL of hybridizing solution containing probe (300 ng/mL) was added to the slides for hybridization at 42 °C overnight. Following 3 washes with PBS containing 0.05% (v/v) Tween-20 (PBST), DAPI (1:800) diluted with PBST was added to stain the nuclei; the slides were then transferred to 24-well culture plates and incubated for 5 min. The slides were then sealed using an anti-fluorescence quenching agent and photographed using a fluorescence microscope (Olympus)[29].

Nuclear/cytosol fractionation

HK2 cells were resuspended in hypotonic buffer A [10 mm HEPES (pH 7.5), 0.5 mm DTT, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$] containing protease inhibitor and RNase inhibitor (N8080119, Thermo Fisher Scientific). The HK2 cells were then incubated on ice for 10 min and centrifuged (1000 × g, 4 °C, 10 min). The supernatant was further centrifuged at 15000 × g for 15 min to obtain the cytoplasmic component. The precipitate was washed twice with hypertonic buffer, resuspended in hypotonic buffer B [10 mm HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mm DTT, 0.5% Nonidet P-40], incubated at 4 °C for 30 min, and then centrifuged at 6000 × g, 4 °C and for 10 min. The precipitate was rinsed once with hypertonic buffer and resuspended in RIPA buffer containing protease inhibitor and RNase inhibitor [50 mm Tris HCl (pH 7.5), 1500 mmol/L KCl, 1% Nonidet P-40, 0.5% sodium hydroxide, 0.1% SDS, 1 mmol/L EDTA, pH 8.0], incubated at 4 °C for 30 min, rotated gently, and then centrifuged at 15000 × g for 20 min. The supernatant contained the nuclei[30].

Dual-luciferase assay

The binding sites of XIST and miR-15b-5p, miR-15b-5p and TLR4 were predicted by the bioinformatics website StarBase (http://starbase.sysu.edu.cn/index.php). The binding site sequences or their mutants were amplified and inserted into the pmiR vector (Huayueyang, Beijing, China) to construct the wild-type (WT) or mutant (MUT) vectors XIST-WT, TLR4-WT, XIST-MUT, and TLR4-MUT. Subsequently, HEK293T cells (Beinuo, Shanghai, China) were cotransfected with mimic NC or miR-15b-5p mimic for 48 h, after which the cells were collected and lysed, and the luciferase activity was detected using a kit (K801-200; Biovision, Mountain View, CA, United States).

RNA immunoprecipitation

The binding of miR-15b-5p to TLR43 and XIST was detected by an RNA immunoprecipitation (RIP) kit (Millipore, MA, United States). HK2 cells were rinsed with precooled PBS, and the supernatant was discarded. The cells were incubated with an equal volume of RIPA lysate (P0013B, Beyotime, Shanghai, China) for 5 min and centrifuged at 4 °C for 10 min to obtain the supernatant. One part of the cell extract was taken as input, and the other part was incubated with the antibody for coprecipitation. RNA was extracted from the sample after detachment with proteinase K and used for qRT–PCR of XIST, miR-15b-5p, and TLR4. The antibodies used in RIP were AGO2 (1:100, ab32381, Abcam, Cambridge, United Kingdom) and rabbit anti-IgG (1:100, ab109489, Abcam, Cambridge, United Kingdom), which was used as the control.

Statistical analysis

The statistical review of the study was performed by a biomedical statistician. SPSS 21.0 (IBM Corp. Armonk, NY, United States) was used to process the data, and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States) was used for mapping. All measurement data were tested for normal distribution and homogeneity of variance and are presented as the mean ± SD. The comparisons among multiple groups were conducted by one-way or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. A probability value of $P < 0.05$ implied a statistically significant difference.

RESULTS

Silencing lncRNA XIST inhibits STZ-induced renal injury in DN rats

An increasing number of studies have focused on the regulatory mechanism of lncRNAs in DN development. XIST is upregulated in acute kidney injury, and inhibiting XIST can alleviate acute kidney injury[31]. However, the regulatory mechanism of XIST in DN requires further exploration.

To study the role of XIST in DN, we first established a DN rat model and detected XIST expression. XIST in the DN group was higher than that in the control group. We then used LV-sh-XIST lentivirus to knock down XIST expression in the model rats ($P < 0.05$, Figure 1A) and detected the renal metabolic and biochemical indices (including KW/BW, FBG, BUN, Cr, and UP 24 h) of the rats. The KW/BW, FBG, BUN, Cr, and UP 24 h values in DN rats were higher than those in control rats, while LV-sh-XIST injected *via* the caudal vein significantly reduced these indices (except for FBG) (all $P < 0.05$, Figure 1B-F). Meanwhile, we detected the histological changes in renal tissue by HE staining, PAS staining, and Masson staining. Compared with the control group, the DN and DN + LV-sh-NC groups had several abnormalities, such as glomerular hypertrophy, basement membrane thickening, mesangial hyperplasia, glomerular epithelial cell swelling, lumen stenosis, inflammatory cell infiltration, and moderate fibrosis. Lentivirus LV-sh-XIST intervention improved all of these histological changes ($P < 0.05$, Figure 1G). Thus, silencing XIST can inhibit renal injury in DN rats.

Silencing XIST inhibits pyroptosis in DN rats

Inflammation is an important pathogenic factor of DN that can lead to RTEC pyroptosis[28]. However, no studies have investigated the effects of XIST on pyroptosis in DN. Here, we detected IL-1β and IL-18 expression in renal tissue. ELISA revealed that IL-1β and IL-18 in the DN group were elevated, but they were lowered after silencing XIST ($P < 0.05$, Figure 2A). Western blot analysis showed that the NLRP3, ASC, cleaved caspase-1, caspase-1, GSDMD, and GSDMD-N levels in the DN group were significantly enhanced, but they were lowered after silencing XIST (all $P < 0.05$, Figure 2B). The caspase-1 immunofluorescence results were consistent with the Western blot results ($P < 0.05$, Figure 2C). These results suggest that silencing XIST in DN can prevent RTEC pyroptosis.

Silencing XIST inhibits pyroptosis in HG-induced HK2 cells

HK2 cells were treated with HG to further study the regulatory effect of XIST on RTEC pyroptosis *in vitro*. qRT–PCR found that HG treatment induced high XIST expression, while XIST was significantly decreased after transfection of a siRNA sequence targeting XIST ($P < 0.05$, Figure 3A). Si-XIST-1, which had the best silencing efficiency, was used in subsequent experiments. Next, we examined the effect of silencing XIST on RTEC pyroptosis after HG treatment. HG treatment significantly promoted the expression of IL-1β and IL-18 (as detected by ELISA), as well as NLRP3, ASC, cleaved caspase-1, caspase-1, GSDMD, and GSDMD-N (as shown by Western blot), while these levels were significantly lower after silencing XIST (all $P < 0.05$, Figure 3B-C). Silencing XIST also annulled the inhibition of HK2 cell viability by HG ($P < 0.05$, Figure 3D) and significantly reduced the release rate of LDH ($P < 0.05$, Figure 3E). Overall, silencing XIST inhibited HG-induced RTEC pyroptosis *in vitro*.

LncRNA XIST binds to miR-15b-5p in DN

To further study the regulatory mechanism of XIST on RTEC pyroptosis in DN, we first used FISH and

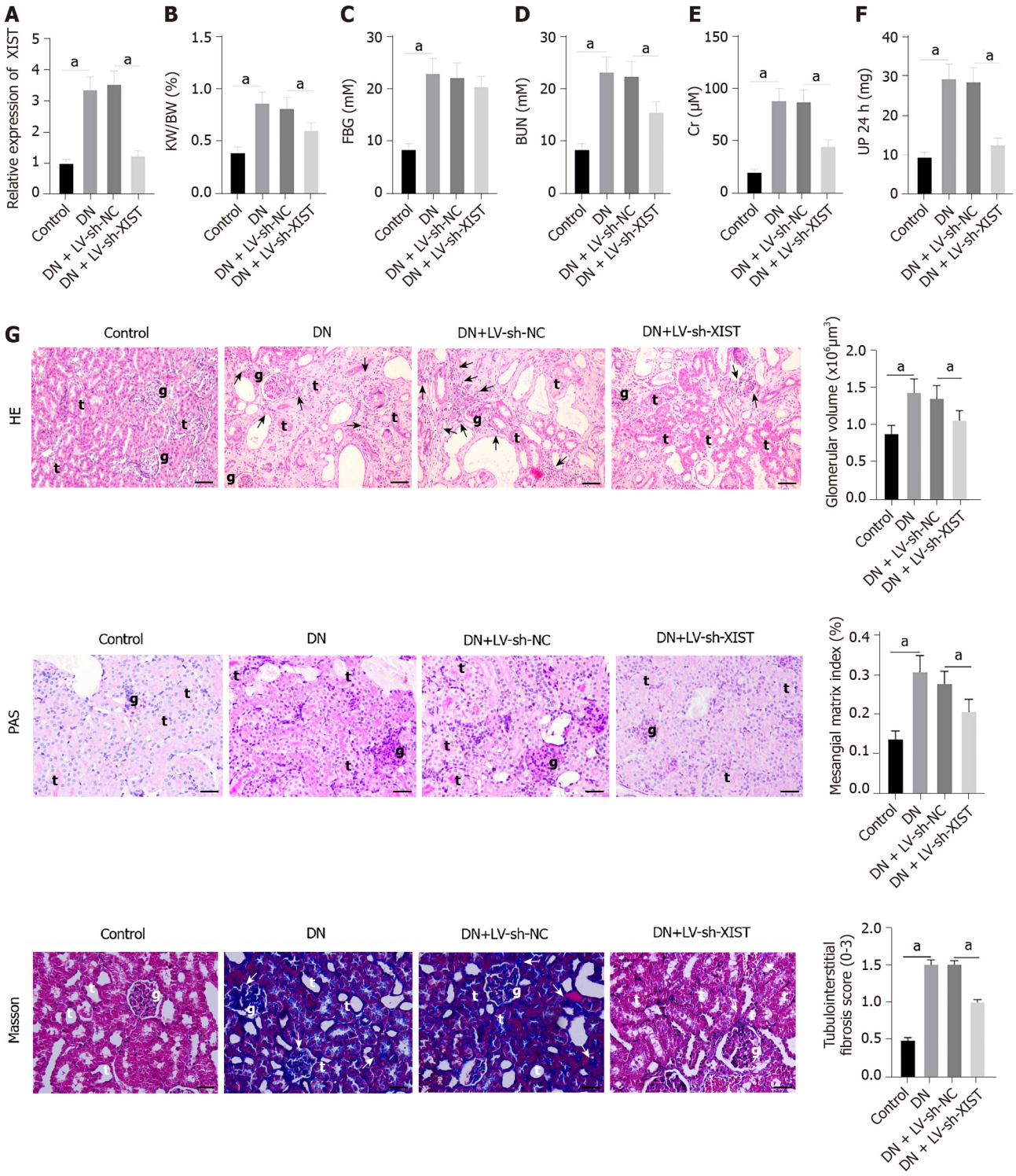

Figure 1 Silencing lncRNA X inactive specific transcript can inhibit renal injury in DN rats. After establishing DN model rats, lentivirus-LV sh- X inactive specific transcript (XIST) was injected into the tail vein to knock down XIST expression *in vivo*. A: XIST expression detected by qRT-PCR; B: Kidney weight/body weight (KW/BW); C: Fasting blood glucose (FBG); D: Blood urea nitrogen (BUN); E: Serum creatinine (Cr); F: Urine protein for 24 h (UP 24 h); G: Histological changes of renal tissue estimated by HE staining, PAS staining and Masson staining; scale bar: 25 μm; arrows indicate inflammatory cell infiltration (black arrows) and fibrosis (white arrows). (g) glomerulus, (t) tubules; n = 8/group. The data were described as mean ± SD and analyzed by one-way ANOVA and Tukey's multiple comparisons test; $^{a}P < 0.05$. DN: Diabetic nephropathy; LV: Lentivirus; XIST: X inactive specific transcript; sh: Short hairpin RNA; HE: Hematoxylin-eosin staining; PAS: Periodic Acid-Schiff stain.

nuclear/cytosolic fractionation to identify that XIST was mainly expressed in the cytoplasm (Figure 4A-B). These results indicated that XIST may play a role in DN *via* a ceRNA loop. StarBase (http://starbase.sysu.edu.cn/index.php) predicted that XIST can bind to miR-15b-5p (Figure 4C). The dual-luciferase experiment ($P < 0.05$, Figure 4D) and RIP experiment verified that XIST could adsorb and bind to miR-15b-5p ($P < 0.05$, Figure 4E). qRT–PCR showed that miR-15b-5p in the DN group was

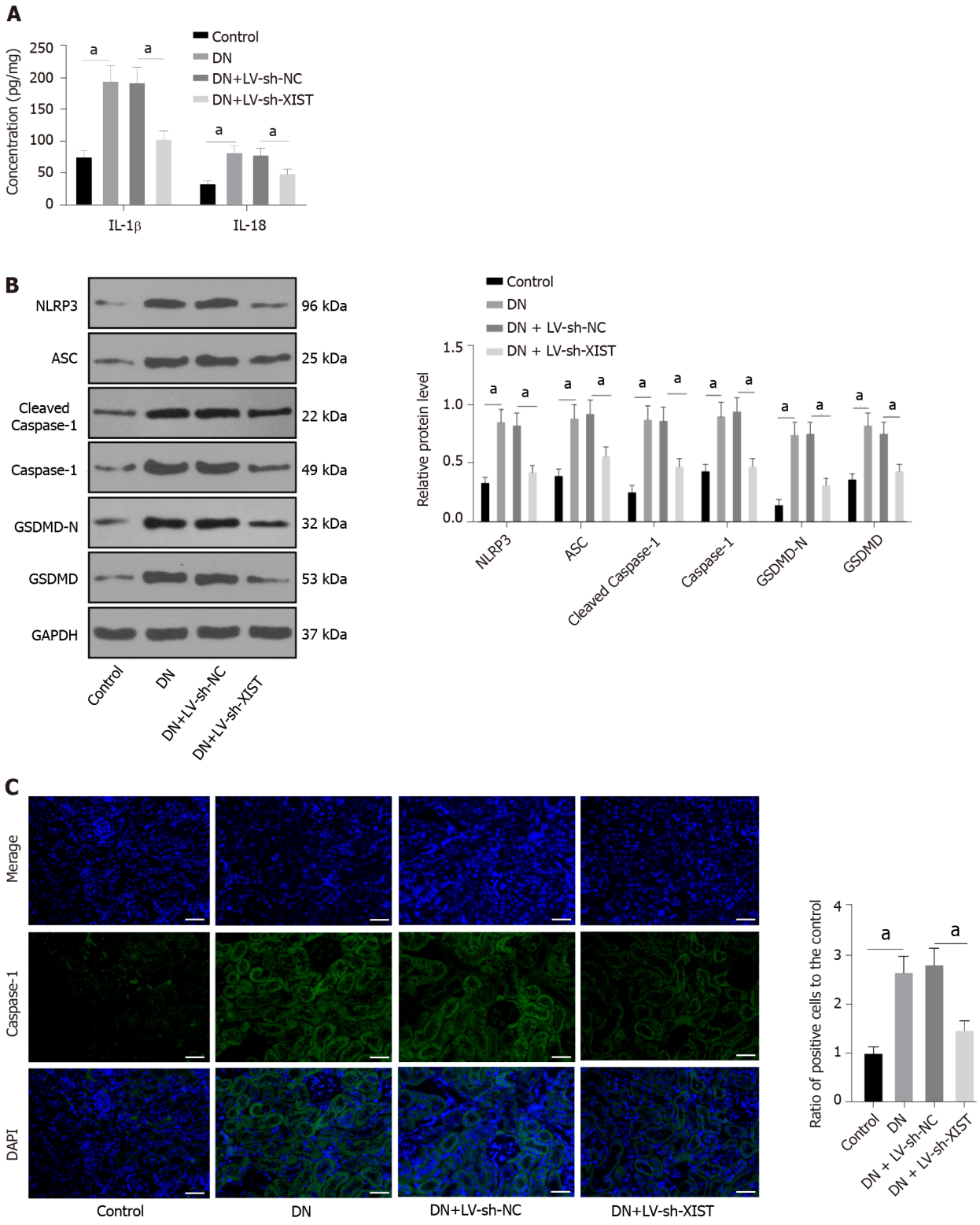

Figure 2 Silencing lncRNA X inactive specific transcript can inhibit renal tubular epithelial cell pyroptosis in diabetic nephropathy rats. After establishing diabetic nephropathy model rats, lentivirus-LV sh- X inactive specific transcript was injected into the tail vein. A: Enzyme-linked immunosorbent assay detected IL-1β and IL-18 expressions; B: Western blot tested the levels of NLR family pyrin domain containing 3, ASC, Cleaved Caspase-1, Caspase-1, GSDMD, and GSDMD-N; C: Immunofluorescence tested the expression of Caspase-1; scale bar: 25 μm; *N* = 8/group. The data were described as mean ± SD and analyzed by one-way ANOVA and Tukey's multiple comparisons test; $^{a}P < 0.05$. DN: Diabetic nephropathy; LV: Lentivirus; XIST: X inactive specific transcript; sh: Short hairpin RNA; NLRP3: NLR family pyrin domain containing 3; ASC: Apoptosis speck-like protein; GSDMD: Gasdermin D; DAPI: 4',6-diamidino-2-phenylindole.

significantly lower and was increased after silencing XIST ($P < 0.05$, Figure 4F). Moreover, miR-15b-5p in HK2 cells was inhibited by HG treatment and was significantly enhanced after silencing XIST ($P < 0.05$, Figure 4G). There was no significant difference in the expression of premiR-15b in rats and cells in each group. These results show that XIST can adsorb and bind to miR-15b-5p to reduce miR-15b-5p

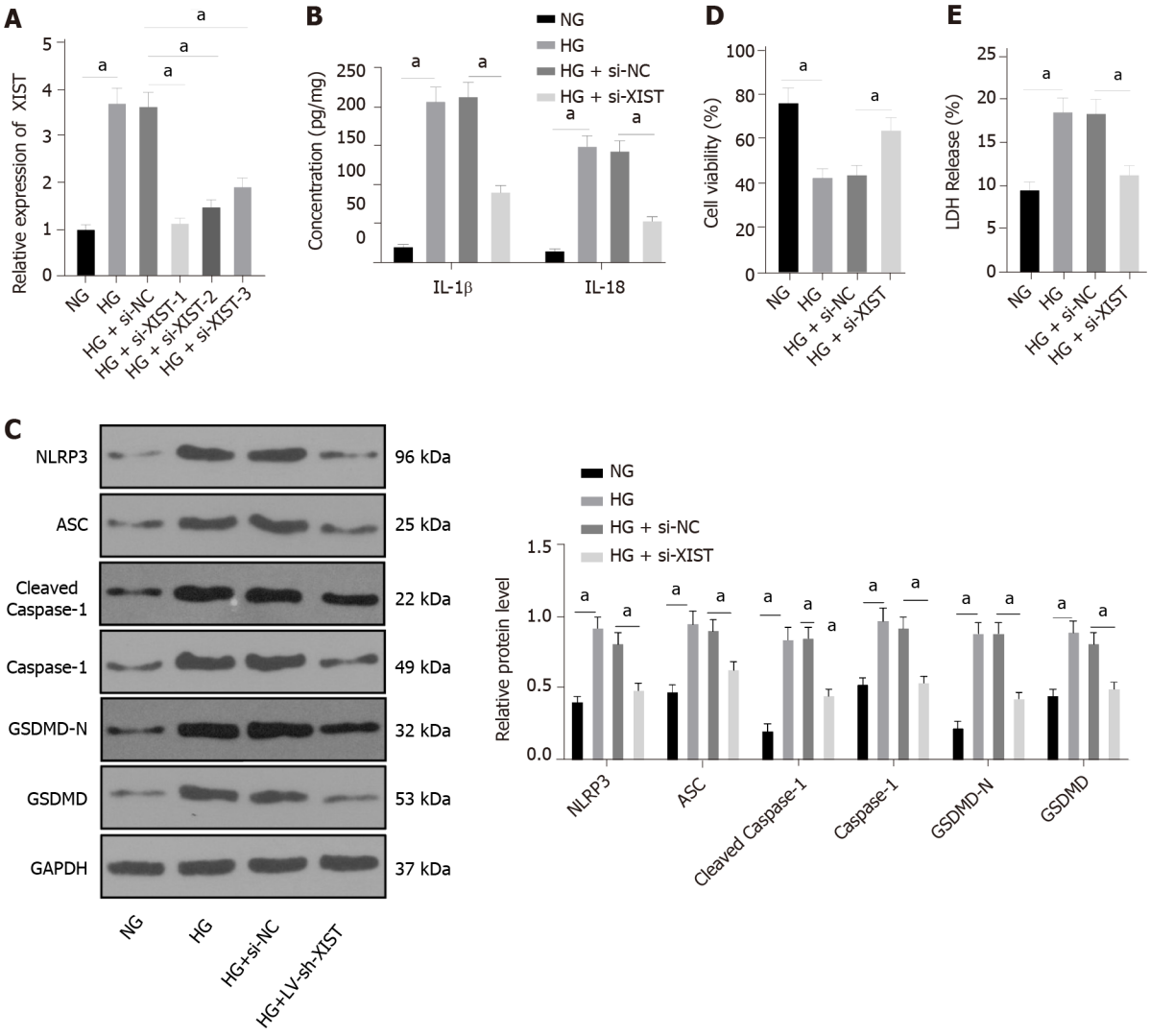

Figure 3 Silencing X inactive specific transcript in diabetic nephropathy can inhibit renal tubular epithelial cell pyroptosis in high glucose-induced HK2 cells. si-NC or si- X inactive specific transcript (XIST) was delivered into HK2 cells treated with high glucose. A: The XIST expression detected by qRT-PCR; B: Enzyme-linked immunosorbent assay detected the expression of IL-1β and IL-18; C: Western blot tested the levels of NLR family pyrin domain containing 3, ASC, Cleaved Caspase-1, Caspase-1, GSDMD, and GSDMD-N; D: Cell viability; E: Lactate dehydrogenase activity. The cell experiment was performed in triplicate. The data were described as mean ± SD and analyzed by one-way ANOVA (A/D-E) or two-way ANOVA (B/C) and Tukey's multiple comparisons test; $^{a}P < 0.05$. NG: Normal glucose; HG: High glucose; si: Small interfering RNA; XIST: X inactive specific transcript; LDH: Lactate dehydrogenase; NLRP3: NLR family pyrin domain containing 3; SC: Apoptosis speck-like protein; GSDMD: Gasdermin D.

expression in DN.

Inhibition of miR-15b-5p in XIST-silenced HK2 cells activates RTEC pyroptosis

Next, we carried out functional rescue experiments to verify the above regulatory mechanism. We silenced both XIST and miR-15b-5p in HG-exposed HK2 cells. First, we detected miR-15b-5p expression by qRT–PCR, which verified the transfection efficiency of the miR-15b-5p inhibitor ($P < 0.05$, Figure 5A). In HG-treated HK2 cells, the miR-15b-5p inhibitor reversed the inhibitory effect of si-XIST on IL-1β and IL-18 (all $P < 0.05$, Figure 5B); it also reversed the expression of NLRP3, ASC, cleaved caspase-1, caspase-1, GSDMD, and GSDMD-N (all $P < 0.05$, Figure 5C). Compared with the si-XIST + inhibitor NC group, the cell viability of the si-XIST + miR-15b-5p inhibitor group was decreased ($P < 0.05$, Figure 5D), while the release rate of LDH was increased ($P < 0.05$, Figure 5E). Taken together, these results indicate that silencing XIST inhibits HG-induced RTEC pyroptosis *in vitro* by promoting miR-15b-5p.

LncRNA XIST sponges miR-15b-5p to inhibit TLR4

To study the downstream mechanism of miR-15b-5p, we utilized the StarBase website (http://starbase.sysu.edu.cn/index.php) to predict that miR-15b-5p can bind to the 3'UTR of TLR4 (Figure 6A). The dual-luciferase assay and RIP assay also showed that TLR4 was the direct target gene of miR-15b-5p ($P < 0.05$, Figure 6B-C). TLR4 expression in renal tissue was assessed by qRT–PCR and Western blot. The results showed that TLR4 in renal tissue of the DN group was higher than that of the control group, and

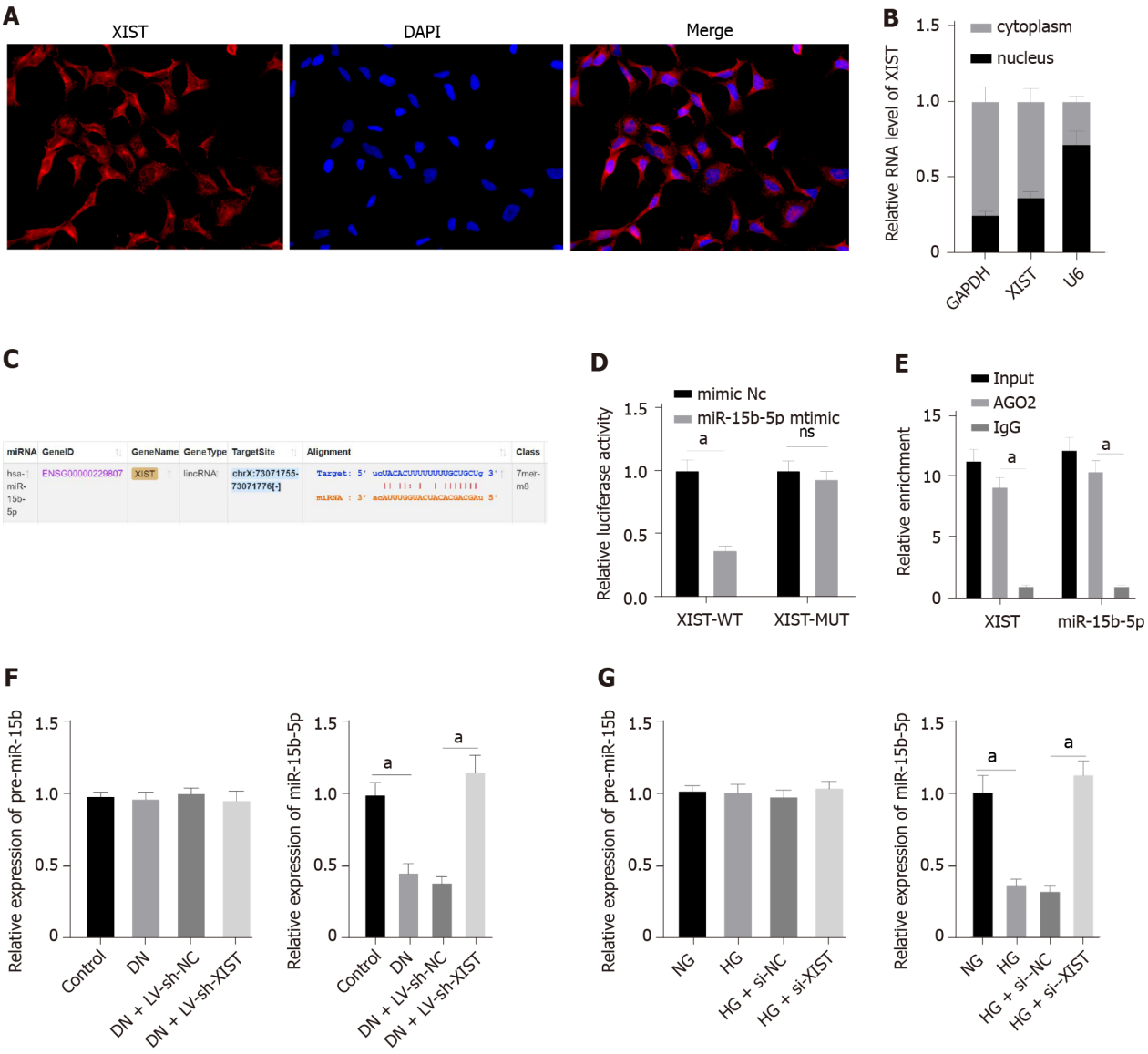

Figure 4 X inactive specific transcript can adsorb and bind to miR-15b-5p to reduce miR-15b-5p expression in diabetic nephropathy. A: Fluorescence in situ hybridization assay (scale bar: 25 μm); B: Nuclear/cytosol fractionation assay verified the subcellular localization of X inactive specific transcript (XIST) in HK2 cells; C: Starbase website (starbase.sysu.edu.cn/index.php) predicted the binding sites of XIST and miR-15b-5p; D: Dual-luciferase experiment and E: RNA immunoprecipitation experiment verified the binding relation of XIST and miR-15b-5p; F: qRT-PCR detected miR-15b-5p and pre-miR-15b expression in renal tissue; G: qRT-PCR detected miR-15b-5p and pre-miR-15b expression in HK2 cells. The cell experiment was performed in triplicate. The data were described as mean ± SD and analyzed by one-way ANOVA (F/G) or two-way ANOVA (D/E) and Tukey's multiple comparisons test; $^{a}P < 0.05$. XIST: X inactive specific transcript; DN: Diabetic nephropathy; DAPI: 4',6-diamidino-2-phenylindole; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; U6: U6 small nuclear RNA; WT: Wild type; MUT: Mutant.

TLR4 was lower after silencing XIST (all $P < 0.05$, Figure 6D-E). Additionally, silencing XIST significantly reversed the TLR4 expression induced by HG, while the miR-15b-5p inhibitor reversed the inhibitory effect of silencing XIST (all $P < 0.05$, Figure 6F-G). In summary, XIST inhibits TLR4 expression by binding to miR-15b-5p.

TLR4 overexpression in XIST-silenced cells causes increased pyroptosis

TLR4 promotes apoptosis by activating NLRP3/caspase-1 in hepatic ischaemia–reperfusion injury[32]. Whether XIST regulates HG-induced RTEC pyroptosis through TLR4 remains to be explored.

To further verify the regulatory effect of XIST on TLR4, we silenced XIST and overexpressed TLR4 in HG-treated HK2 cells and detected the TLR4 Levels. TLR4 was significantly overexpressed ($P < 0.05$, Figure 7A-B). Compared with the si-XIST + oe-NC group, TLR4 overexpression promoted IL-1β and IL-18 expression and increased the levels of NLRP3, ASC, cleaved caspase-1, caspase-1, GSDMD, and GSDMD-N (all $P < 0.05$, Figure 7C-D). Furthermore, TLR4 overexpression decreased cell viability ($P < 0.05$, Figure 7E) and increased the release rate of LDH ($P < 0.05$, Figure 7F). In summary, silencing XIST inhibits HG-induced RTEC pyroptosis by inhibiting TLR4.

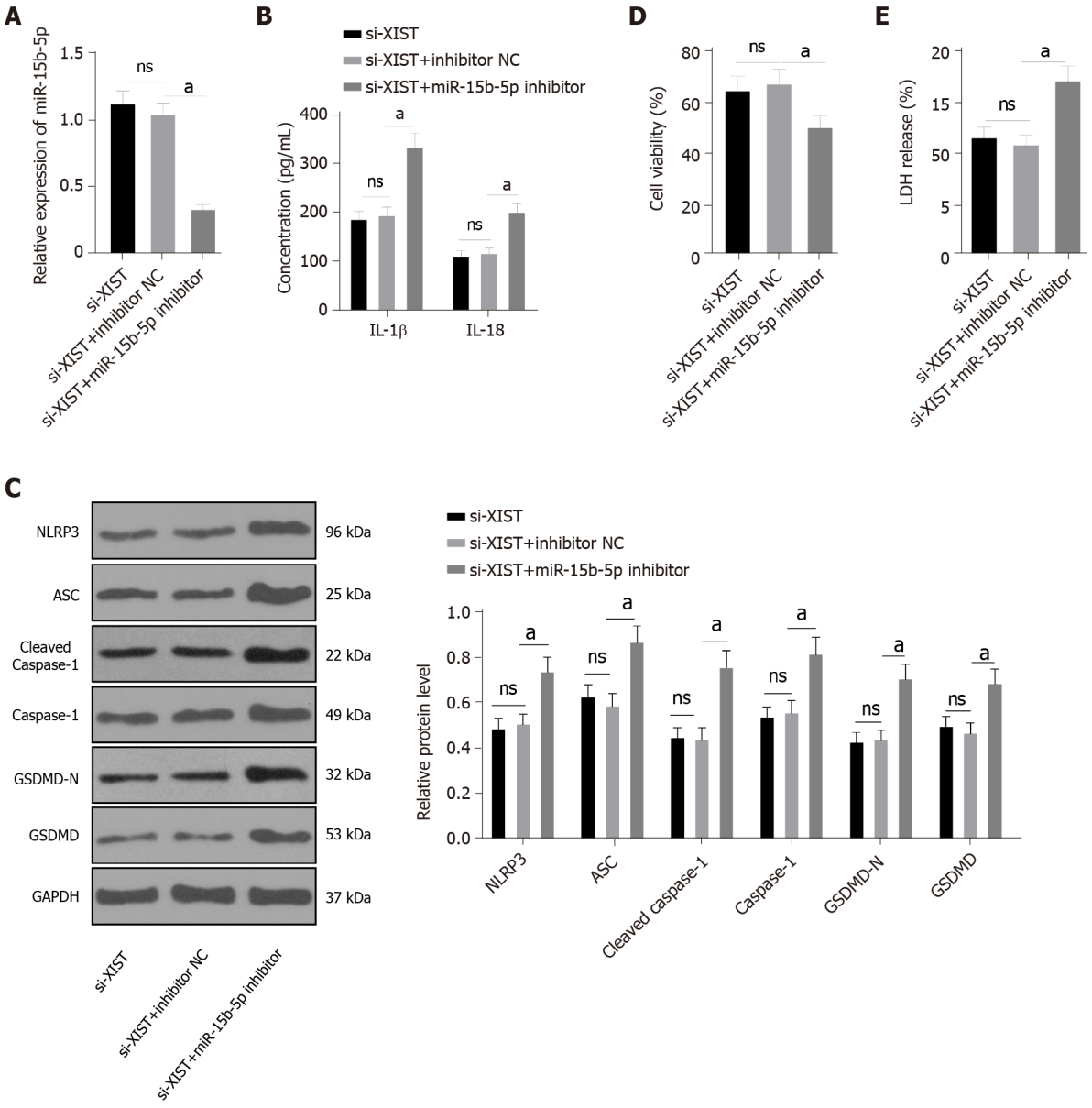

Figure 5 Inhibition of miR-15b-5p in X inactive specific transcript -silenced HK2 cells activates renal tubular epithelial cell pyroptosis. In HG-treated HK2 cells, both X inactive specific transcript (XIST) and miR-15b-5p were silenced. A: qRT-PCR verified the transfection efficiency of miR-15b-5p inhibitor; B: Enzyme-linked immunosorbent assay detected the expression of IL-1β and IL-18; C: Western blot tested the levels of NLR family pyrin domain containing 3, ASC, Cleaved Caspase-1, Caspase-1, GSDMD, and GSDMD-N; D: Cell viability; E: Lactate dehydrogenase activity. The cell experiment was performed in triplicate. The data were described as mean ± SD and analyzed by one-way ANOVA (A/D-E) or two-way ANOVA (B/C) and Tukey's multiple comparisons test; $^{a}P < 0.05$. LDH: Lactate dehydrogenase; XIST: X inactive specific transcript; si: Small interfering RNA; NLRP3: NLR family pyrin domain containing 3; ASC: Apoptosis speck-like protein; GSDMD: Gasdermin D.

DISCUSSION

As the most prevalent, disastrous, and costly complication of diabetes, DN occurs in approximately 30% to 40% of diabetic patients, representing a considerable public health problem[33]. Pyroptosis and the subsequent inflammatory response are dominant events in DN pathogenesis[15]. This study found that DN induces RTEC pyroptosis by activating NLRP3/caspase-1 and inducing high XIST expression. Silencing XIST promoted the targeted inhibition of TLR4 by miR-15b-5p by upregulating miR-15b-5p, thus inhibiting TLR4 and finally alleviating renal injury in DN rats by inhibiting NLRP3/caspase-1-mediated RTEC pyroptosis (Figure 8).

XIST silencing has been shown to alleviate certain biological processes and inflammation in HG-treated human mesangial cells (HMCs)[21]. XIST was found to be highly expressed in renal tissue of DN patients, STZ-treated DN mice, and HG-exposed HK-2 cells[22]. Consistently, the STZ (50 mg/kg)-induced DN rat model in our study showed the upregulation of XIST. XIST expression was downregulated by LV-sh-XIST lentivirus, and renal metabolic and biochemical indices were detected. The results showed that LV-sh-XIST significantly reduced the values of KW/BW, BUN, Cr, and UP 24 h. FBG is a

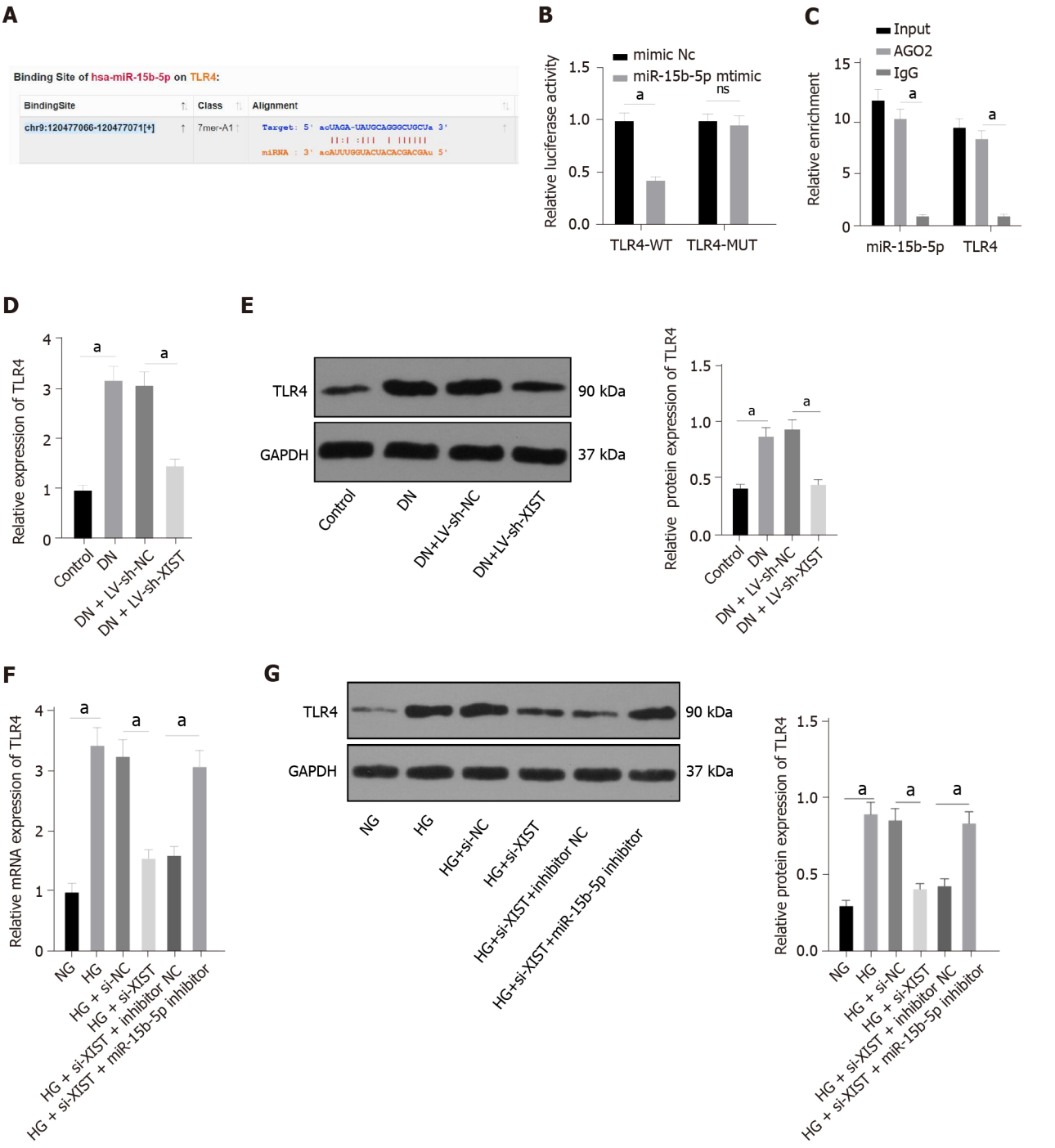

Figure 6 Toll like receptor 4 expression is recovered by using miR-15b-5p inhibitor. A: Starbase website (starbase.sysu.edu.cn/index.php) predicted the binding sites of miR-15b-5p and toll like receptor 4 (TLR4); B: Dual-luciferase experiment and C: RNA immunoprecipitation experiment verified the binding relation of miR-15b-5p and TLR4; D: qRT-PCR detected TLR4 expression in renal tissues; E: Western blot detected TLR4 level in renal tissues; F: qRT-PCR detected TLR4 expression in HK2 cells; G: Western blot detected TLR4 level in HK2 cells. The cell experiment was performed in triplicate. The data were described as mean ± SD and analyzed by one-way ANOVA (D-G) or two-way ANOVA (B/C) and Tukey's multiple comparisons test; $^{a}P < 0.05$. WT: Wild type; MUT: Mutant; TLR4: Toll like receptor 4; DN: Diabetic nephropathy; LV: Lentivirus; XIST: X inactive specific transcript; NG: Normal glucose; HG: High glucose; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

highly related indicator of diabetes. XIST silencing can improve the symptoms of diabetic nephropathy but cannot completely cure diabetes. Correspondingly, our results showed that FBG was not restored after XIST silencing. One study showed that a markedly elevated FBG content in the serum of DN rats indicated seriously disordered glucose metabolism; the DN rats also presented elevated BUN and Cr levels, which were associated with impaired renal filtration function[34]. As previously noted, the metabolic burden caused by hyperglycaemia can result in metabolic injuries, such as tissue inflammation, glomerular filtration, tubular hypertrophy, and tissue fibrosis[35]. In the current study, histological staining of renal tissue demonstrated that the DN rats showed glomerular hypertrophy,

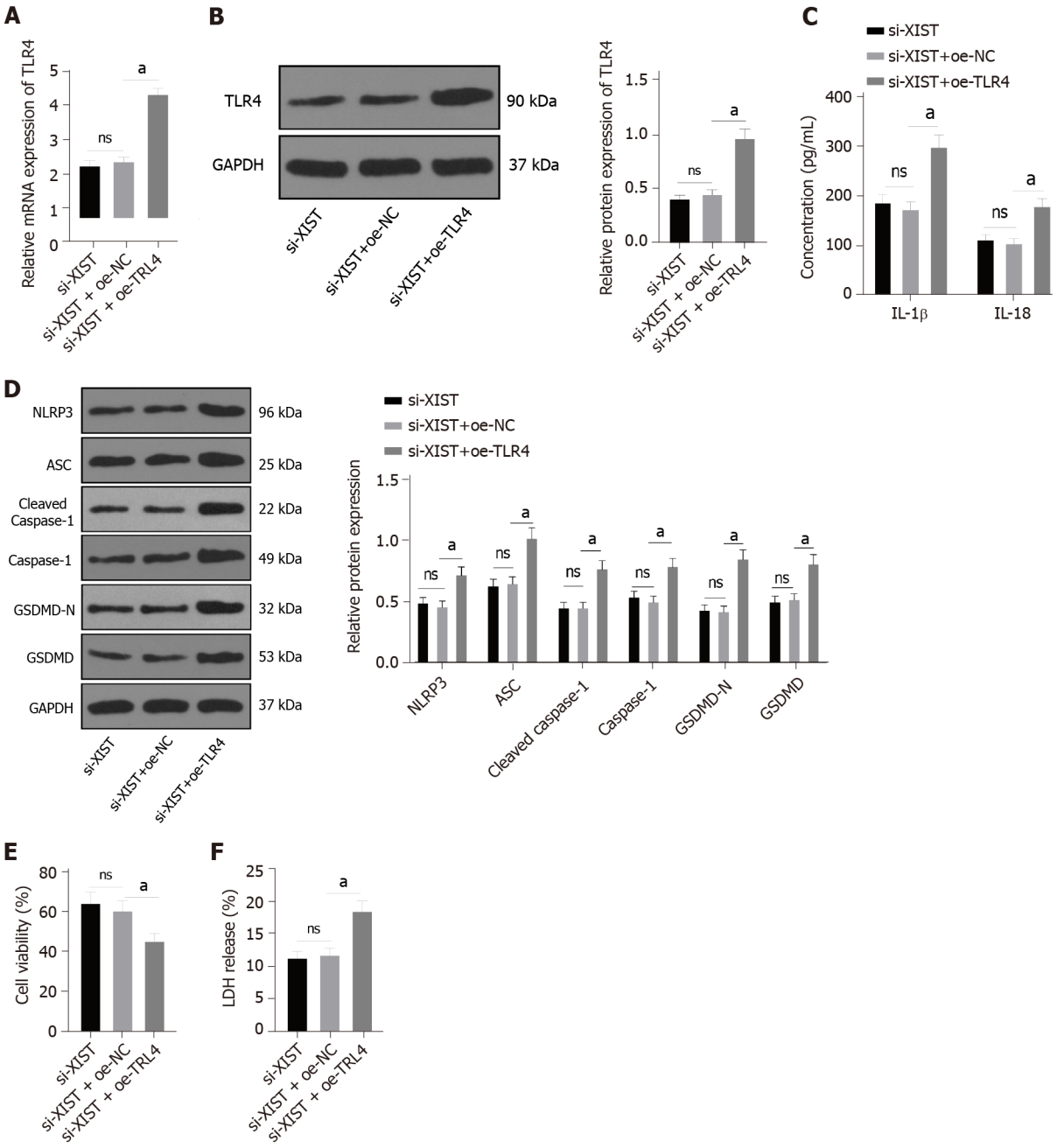

Figure 7 Toll like receptor 4 overexpression in X inactive specific transcript-silenced cells causes increased pyroptosis. In HG-treated HK2 cells, X inactive specific transcript (XIST) was silenced and toll like receptor 4 (TLR4) was overexpressed. A and B: The expression of TLR4 levels detected by qRT-PCR and Western blot; C: Enzyme-linked immunosorbent assay detected the expression of IL-1β and IL-18; D: Western blot tested the levels of NLR family pyrin domain containing 3, ASC, Cleaved Caspase-1, Caspase-1, GSDMD, and GSDMD-N; E: Cell viability; F: LDH activity. The cell experiment was performed in triplicate. The data were described as mean ± SD and analyzed by one-way ANOVA (A/B/E/F) or two-way ANOVA (C/D) and Tukey's multiple comparisons test; $^{a}P < 0.05$. XIST: X inactive specific transcript; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; LDH: Lactate dehydrogenase; NLRP3: NLR family pyrin domain containing 3; ASC: Apoptosis speck-like protein; GSDMD: Gasdermin D.

basement membrane thickening, mesangial hyperplasia, glomerular epithelial cell swelling, lumen stenosis, inflammatory cell infiltration, and fibrosis; however, LV-sh-XIST intervention improved these histological changes. Preventing glomerular hypertrophy and attenuating glomerular hyperfiltration may have therapeutic potential for DN[35]. Consistently, prior work suggests that XIST knockdown alleviates acute kidney injury[31]. These results suggest that silencing lncRNA XIST can inhibit renal injury in DN rats.

Inflammation and RTEC pyroptosis are hallmarks of tubular cell damage and renal functional deterioration in kidney injury[36]. The urinary level of the inflammatory mediator IL-18 has been reported to be positively linked with DN progression[37]. The participation of pyroptosis in DN progression has recently attracted much attention[38,28], and we investigated inflammation and RTEC pyroptosis-related factors. Our results revealed that the IL-1β and IL-18 levels in DN rats were elevated, but they

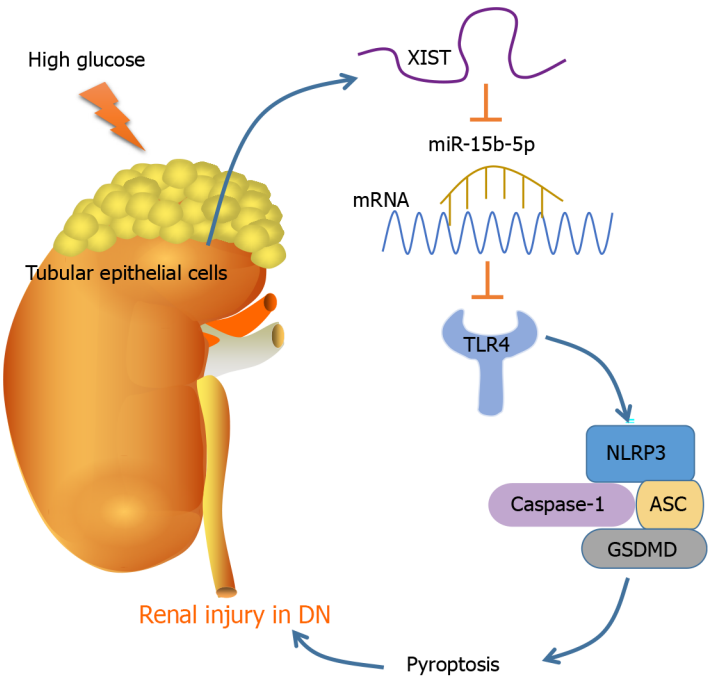

Figure 8 Mechanism diagram. STZ and HG treatment induced high expression of X inactive specific transcript (XIST); silencing XIST promoted the targeted inhibition of miR-15b-5p on toll like receptor 4 (TLR4) by upregulating miR-15b-5p, thereby inhibiting TLR4, and ultimately alleviating the renal injury of diabetic nephropathy by inhibiting NLRP3/Caspase-1-mediated RTEC pyroptosis. mRNA: messenger RNA; DN: Diabetic nephropathy; NLRP3: NLR family pyrin domain containing 3; ASC: Apoptosis speck-like protein; GSDMD: Gasdermin D.

were lower after silencing XIST. Western blot and caspase-1 immunofluorescence showed that the NLRP3, ASC, caspase-1, and GSDMD levels in the DN group were enhanced, but they were lower after silencing XIST, which was highly consistent with previous studies[39,34]. NLRP3 inflammasome activation is related to cell pyroptosis[40]; therefore, the inhibition of NLRP3 inflammatory bodies may be a potential option for DN treatment[41]. To further study the regulatory effect of XIST on pyroptosis, HK2 cells were treated with HG to further study the regulatory effect of XIST on RTEC pyroptosis *in vitro*. Our results revealed that silencing XIST inhibited HG-induced RTEC pyroptosis *in vitro*, reversed the inhibitory effect of HG on HK2 cell viability, and reduced the release rate of LDH. Podocytes exposed to 30 mM HG showed significantly elevated levels of caspase-11, GSDMD-N, IL-1β, and IL-18, indicating that pyroptosis is activated under hyperglycaemic conditions and during DN development [14]. Collectively, the results indicate that silencing XIST in DN can inhibit HG-induced RTEC pyroptosis.

To further study the regulatory mechanism of XIST on RTEC pyroptosis in DN, we used FISH and nuclear/cytosolic fractionation to identify that XIST was mainly expressed in the cytoplasm. These results suggest that XIST plays a role in DN by regulating ceRNAs. StarBase predicted that XIST can bind to miR-15b-5p, which was validated by dual-luciferase experiments and RIP. qRT-PCR showed that miR-15b-5p in the DN models was significantly lower but increased after silencing XIST. Accordingly, miR-15b-5p was found to be downregulated in the serum of DN patients and in HG-treated HMCs[42]. XIST can adsorb and bind to miR-15b-5p to reduce miR-15b-5p expression in DN. We did not investigate whether XIST undergoes nuclear translocation in the DN models or in HG-treated HK-2 cells. We will focus on this question in future research to provide a more comprehensive mechanism for the role of XIST in DN.

We next carried out functional rescue experiments to verify the postulated regulatory mechanism. XIST and miR-15b-5p were both silenced in HG-treated HK2 cells. Accordingly, the miR-15b-5p inhibitor significantly reversed the inhibitory effect of si-XIST on IL-1β, IL-18, NLRP3, ASC, caspase-1, and GSDMD; the cell viability was decreased, while the release rate of LDH was significantly increased. Similarly, a previous study revealed that forced expression of miR-15b-5p restrained HG-triggered apoptosis of podocytes and inflammatory responses and repressed oxidative stress damage[43]. The miR-15b-5p mimic repressed the viability and inflammation of HG-treated HMCs, as previously shown [42]. The findings show that silencing XIST inhibits HG-induced RTEC pyroptosis *in vitro* by promoting miR-15b-5p. As yet, there have been no studies on the correlation between miR-15b-5p and RTEC pyroptosis in DN, indicating the novelty of our findings.

To study the downstream mechanism of miR-15b-5p, the StarBase website was used to predict that miR-15b-5p can bind to TLR4, which was validated by a dual-luciferase assay and RIP assay. qRT–PCR and Western blotting showed that TLR4 in the DN models was significantly enhanced but was lower

after silencing XIST. The miR-15b-5p inhibitor mitigated the inhibition of TLR4 when XIST was silenced. In summary, XIST inhibits TLR4 by binding to miR-15b-5p. RTEC pyroptosis-induced tubular injury is accompanied by upregulated TLR4 and GSDMD in DN patients[44]. To further verify that the regulation of RTEC pyroptosis by XIST is realized through TLR4, we silenced XIST and overexpressed TLR4 in HG-treated HK2 cells. As expected, TLR4 overexpression promoted IL-1β, IL-18, NLRP3, ASC, cleaved caspase-1, caspase-1, GSDMD, and GSDMD-N; decreased cell viability; and increased the release rate of LDH. The results of several studies support our findings. TLR4 promotes apoptosis by activating NLRP3/caspase-1[32]. TLR4 depletion inhibits microglial pyroptosis to promote motor function recovery after spinal cord injury[45]. The TLR4 inhibitor TAK-242 induced GSDMD-mediated pyroptosis in HG-exposed cells[44]. In summary, silencing XIST inhibits HG-induced RTEC pyroptosis by inhibiting TLR4.

CONCLUSION

Overall, our results indicate that silencing XIST ultimately relieves renal injury in DN by inhibiting NLRP3/caspase-1-mediated RTEC pyroptosis *via* the ceRNA network of eXIST/miR-15b-5p/TLR4. However, the mechanism of abnormal XIST and miR-15b-5p expression in renal injury in DN has not been comprehensively studied and will be further explored in our future studies. There are many pyroptosis regulatory mechanisms, among which the classical caspase-1 inflammatory body pathway is the most studied. This pathway includes NLRP1, NLRP3, and other nod-like receptor family inflammatory bodies. Here, we investigated the classical caspase-1 pathway. Many studies have shown that the caspase-11 pathway plays an important role in inflammation-related diseases[14,46,47]. We will further explore whether XIST has a regulatory effect on the caspase-11 pathway in future research.

ARTICLE HIGHLIGHTS

Research background

Diabetes is a metabolic disease characterized by hyperglycemia. Chronic hyperglycemia can lead to chronic damage and dysfunction of various tissues and organs, such as eyes, kidneys, heart, blood vessels, and nerves. Various complications caused by diabetes are unavoidable problems in the treatment of diabetes mellitus, including diabetic nephropathy (DN). Understanding the molecular regulation mechanism of DN during renal injury is helpful to the treatment of DN.

Research motivation

Cell pyroptosis is a programmed cell death pattern and plays a key role in DN. However, the regulation mechanism of cell pyroptosis in DN has not been studied clearly. Therefore, our research will help to reveal the role of cell pyroptosis in DN.

Research objectives

Long noncoding RNA X inactive specific transcript (LncRNA XIST) was taken as the main object of our research to explore the molecular mechanism of XIST in pyroptosis of renal tubular epithelial cells (RTECs). We found that XIST comparatively bound to microRNA (miR)-15b-5p to regulate Toll like receptor 4 (TLR4) expression and promote RTEC pyroptosis to participate in kidney injury in DN. Our research results provide a new theoretical basis for the treatment of DN.

Research methods

To study the mechanism of XIST in the pathogenesis of DN-induced pyroptosis, we established DN rat models and high glucose (HG)-induced cell models. By detecting and intervening in the expression of XIST in animal models and cell models, we observed the pathological and representational changes of the models, and collected and analyzed the experimental data. Our research methods conform to science and are carried out strict experimental operation according to the experimental principle, and the results are representative.

Research results

Through experiments, we found that lncRNA XIST was highly expressed in the DN models, and XIST increased the expression of TLR4 as a competing endogenous RNA by competing with miR-15-5p. Inhibiting the expression of XIST repressed the expression of TLR4 by upregulating miR-15-5p, and effectively improved the pyroptosis of RTECs induced by DN. Our findings explain the regulatory mechanism of lncRNA XIST in the pathogenesis of DN-induced RTEC pyroptosis. However, our results have not yet been clinically validated and further exploration is warranted in clinical transformation.

Research conclusions

This study for the first time revealed that lncRNA XIST can enhance the expression of TLR4 through competitively binding to miR-15-5p and promote the pyroptosis of RTECs induced by DN. No new methods were used during the study.

Research perspectives

In the future, we will explore more mechanisms for other targeted miRNAs in the downstream of lncRNA XIST, and we will also study the clinical transformation of XIST/miR-15-5p/TLR4 in DN.

FOOTNOTES

Author contributions: Xu J, Wang Q, and Ren YP designed the research study; Song YF, Xu XH, Zhu H and Chen PD performed the research; Wang Q, Liang L and Xu XH analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

Supported by Natural Science Foundation of Shenzhen University General Hospital (SUGH2020QD011).

Institutional animal care and use committee statement: All experiments and procedures were conducted following the laboratory animal care and use guidelines.

Conflict-of-interest statement: For this activity, the authors have no conflicts to declare.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu RR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 April 15; 13(4): 376-386

DOI: 10.4239/wjd.v13.i4.376

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Risk factors for mortality within 6 mo in patients with diabetes undergoing urgent-start peritoneal dialysis: A multicenter retrospective cohort study

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Specialty type: Urology and nephrology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Darenskaya MA, Russia; Sateesh J, India

Received: November 9, 2021
Peer-review started: November 9, 2021
First decision: January 12, 2022
Revised: January 20, 2022
Accepted: March 16, 2022
Article in press: March 16, 2022
Published online: April 15, 2022

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Abstract

BACKGROUND

The risk of early mortality of patients who start dialysis urgently is high; however, in patients with diabetes undergoing urgent-start peritoneal dialysis (USPD), the risk of, and risk factors for, early mortality are unknown.

AIM

To identify risk factors for mortality during high-risk periods in patients with diabetes undergoing USPD.

METHODS

This retrospective cohort study enrolled 568 patients with diabetes, aged ≥ 18 years, who underwent USPD at one of five Chinese centers between 2013 and 2019. We divided the follow-up period into two survival phases: The first 6 mo of USPD therapy and the months thereafter. We compared demographic and

baseline clinical data of living and deceased patients during each period. Kaplan-Meier survival curves were generated for all-cause mortality according to the New York Heart Association (NYHA) classification. A multivariate Cox proportional hazard regression model was used to identify risk factors for mortality within the first 6 mo and after 6 mo of USPD.

RESULTS

Forty-one patients died within the first 6 mo, accounting for the highest proportion of mortalities (26.62%) during the entire follow-up period. Cardiovascular disease was the leading cause of mortality within 6 mo (26.83%) and after 6 mo (31.86%). The risk of mortality not only within the first 6 mo but also after the first 6 mo was higher for patients with obvious baseline heart failure symptoms than for those with mild or no heart failure symptoms. Independent risk factors for mortality within the first 6 mo were advanced age [hazard ratio (HR: 1.908; 95%CI: 1.400-2.600; P < 0.001), lower baseline serum creatinine level (HR: 0.727; 95%CI: 0.614-0.860; P < 0.001), higher baseline serum phosphorus level (HR: 3.162; 95%CI: 1.848-5.409; P < 0.001), and baseline NYHA class III-IV (HR: 2.148; 95%CI: 1.063-4.340; P = 0.033). Independent risk factors for mortality after 6 mo were advanced age (HR: 1.246; 95%CI: 1.033-1.504; P = 0.022) and baseline NYHA class III-IV (HR: 2.015; 95%CI: 1.298-3.130; P = 0.002).

CONCLUSION

To reduce the risk of mortality within the first 6 mo of USPD in patients with diabetes, controlling the serum phosphorus level and improving cardiac function are recommended.

Key Words: Peritoneal dialysis; Urgent start; Diabetes mellitus, Mortality within the first 6 mo; Risk factor; End-stage renal disease

Core Tip: The first 6 mo after the initiation of urgent-start peritoneal dialysis is a high-risk period. We identified the following as risk factors for mortality within the first 6 mo in urgent-start peritoneal dialysis recipients with diabetes: Advanced age, lower baseline serum creatinine level, higher baseline phosphorus level, and baseline New York Heart Association class III-IV.

Citation: Cheng SY, Yang LM, Sun ZS, Zhang XX, Zhu XY, Meng LF, Guo SZ, Zhuang XH, Luo P, Cui WP. Risk factors for mortality within 6 mo in patients with diabetes undergoing urgent-start peritoneal dialysis: A multicenter retrospective cohort study. *World J Diabetes* 2022; 13(4): 376-386
URL: https://www.wjgnet.com/1948-9358/full/v13/i4/376.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i4.376

INTRODUCTION

End-stage renal disease (ESRD) requiring dialysis is a global health problem[1,2]. Patients with late-stage chronic kidney disease—who often delay visiting a doctor owing to economic difficulties or other reasons—often need to start dialysis urgently without any preparation[3]. Several studies have documented the safety and feasibility of urgent-start peritoneal dialysis (USPD)[4-7]. USPD has several benefits over urgent-start hemodialysis (USHD), including better quality of life, better preservation of residual kidney function, and cost savings[4,8-11].

Studies on patients undergoing peritoneal dialysis have shown that those with diabetes mellitus (DM) have a poorer prognosis than do those without DM, in addition to a poorer survival rate owing to the high prevalence of cardiovascular diseases[12,13]. Currently, there is only a small, single-center study published on USPD in ESRD patients with diabetes[14]; it mainly compares the characteristics and complications between patients with diabetes treated with USPD and USHD. However, it does not identify the risk factors for mortality in patients with diabetes undergoing USPD.

The first 6 mo after the initiation of urgent dialysis is a high-risk period[5]. Patients with diabetes undergoing USPD are critically ill; hence, we speculated that the risk of mortality within the first 6 mo in these patients is high. Additionally, as the patient's peritoneal dialysis treatment progresses, the overall patient condition tends to be stable; therefore, we deliberated that the risk factors for mortality within the first 6 mo may be different from those for mortality after 6 mo in patients with diabetes undergoing USPD. However, the distribution of mortalities over time in patients with diabetes undergoing USPD has not been reported, and the risk factors for mortality within the first 6 mo in these

patients are not clear. This study examines both the occurrence of and the risk factors for mortality within the first 6 mo of USPD initiation in patients with diabetes.

MATERIALS AND METHODS

Patients and study design

We screened patients with ESRD who underwent USPD between January 1, 2013 and December 31, 2019 at the following five hospitals: The Second Hospital of Jilin University, Second Part of the First Hospital of Jilin University, Jilin Central Hospital, Jilin First Automobile Work General Hospital, and Xing'anmeng People's Hospital. Patients with incomplete data, those aged < 18 years, and those without diabetes were excluded. All patients were followed up until mortality, kidney transplantation, technical failure, or the follow-up cutoff date (June 30, 2020). All patients were informed about renal replacement therapy modalities. Although experienced nephrologists guided the choice of modality, the final choice was made by the patient.

This retrospective study was approved by the Ethics Committee of the Second Hospital of Jilin University (design No. 2020031). To identify risk factors for mortality within the first 6 mo of USPD in patients with diabetes, we divided the follow-up time into two survival periods: The first 6 mo and the months thereafter. We compared demographic and baseline clinical data of patients who were living or deceased during each period. To highlight the characteristics of the patients deceased within the first 6 mo, we compared the causes of, and risk factors for, mortality after 6 mo to those of mortality within the first 6 mo.

Dialysis prescription

In the present study, automated peritoneal dialysis (APD) and continuous ambulatory peritoneal dialysis (CAPD) were the two modes of peritoneal dialysis. Peritoneal dialysis for each patient was prescribed based on fluid overload, uremia, hyperkalemia, and acid-base imbalance. A low-volume abdominal cavity (0.5-1.0 L) was initially obtained with the patient in the supine position to avoid dialysate leakage, and the volume was progressively increased to 2 L per cycle within 2 wk. The number of cycles per day was 3-4 for CAPD and 6-9 for APD. The dialysis procedure was performed by a peritoneal dialysis nurse until the patient and/or caregiver could independently perform the process. The patients were followed up every 3-6 mo and peritoneal dialysis dose was adjusted to keep the total Kt/V urea above 1.70 or creatinine clearance above 50 L/week/1.73 m^2.

Data collection and definitions

We collected the following data: (1) Basic information, including sex, age, cardiac function classification, and comorbidities such as diabetes, cerebrovascular disease, hypertension, and tumors; (2) Baseline (before peritoneal dialysis within 2 d) laboratory indicators, including hemoglobin, blood albumin, blood white cells, blood phosphorus, blood calcium, blood potassium, blood creatinine, and blood sodium; and (3) Clinical outcomes, including mortality, technical failure, kidney transplantation, and continued dialysis. USPD was commenced within 2 wk of catheter insertion[15]. Technical failure was considered a transition to hemodialysis and its administration to the patient for at least 1 mo[16]. Cardiovascular events included myocardial infarction, stroke, heart failure, unstable angina, peripheral vascular events, fatty pulmonary embolism, sudden mortality, and unknown mortality caused by cardiovascular disease[17]. In accordance with the New York Heart Association (NYHA) categorization [18], patients without symptoms of heart failure were classified as class 0, whereas those with occasional, effort dyspnea were classified as class I; consistent with the traditional classification of cardiac function class, class II was characterized with mildly limited physical activity and general activity that can cause symptoms of heart failure, class III with obviously limited heart function and mild physical lower-than-general activities that can cause symptoms of heart failure, and class IV with symptoms of heart failure that can occur in a resting state.

Statistical analyses

Baseline characteristics are expressed as median (interquartile range) for continuous data and frequency and percentage for categorical data. For comparisons between groups, the rank-sum test was used for continuous variables, and the chi-square test or exact probability test was used for categorical variables. Kaplan-Meier curves were used to compare the survival rates of patients with different cardiac function classes. A Cox proportional hazard regression model was used to identify the risk factors for mortality during different periods of follow-up. The censored data included switching to HD, renal transplantation, technical failure, loss to follow-up, or still at our PD centers during each period. Additionally, for each selected period, mortalities after the period were censored. Factors with $P < 0.1$ in a univariate analysis were included in the multivariate analysis. Statistical significance was set at $P < 0.05$. SPSS 24.0 software (IBM Corp., Armonk, NY, United States) was used for data analysis, and GraphPad 8.0 software (GraphPad Software, San Diego, CA, United States) was used for plotting.

The statistical methods of this study were reviewed by Jin LN from School of Public Health, Jilin University, Changchun, Jilin, China.

RESULTS

Mortalities within the first 6 mo vs. after 6 mo

In this study, we screened 1751 patients undergoing USPD in the aforementioned five centers between 2013 and 2019, of which, we ultimately included 568 patients with diabetes undergoing USPD (Figure 1). Figure 2 shows the mortality proportions of patients with diabetes for the entire follow-up period calculated at 6-mo intervals after the initiation of USPD. As shown, the highest proportion (26.62%) of mortalities occurred between 0 and 6 mo. In the first 6 mo, 41 people died, with a mortality rate of 7.2%. A total of 113 people died after 6 mo of USPD, with a mortality rate of 22.38%.

Demographic and clinical characteristics of included patients

Patient age and blood glucose level were significantly higher in patients who died within the first 6 mo than in those who survived the first 6 mo ($P < 0.001$, $P = 0.011$, respectively). The patients who died within the first 6 mo had a lower proportion of those with NYHA class 0-II and a much higher proportion of those with NYHA class III-IV than did patients who survived the first 6 mo ($P = 0.009$) (Table 1).

Among the patients who were still followed at our PD centers after the first 6 mo, those who died after 6 mo had more advanced age ($P = 0.001$) and lower levels of baseline serum creatinine and serum phosphorus ($P = 0.009$, $P = 0.001$, respectively) than did those who survived throughout follow-up. Compared with the patients who died after 6 mo of USPD, those who survived throughout the follow-up period included a lower proportion of patients with NYHA class III-IV and a higher proportion of patients with NYHA class 0-II ($P = 0.026$) (Table 1).

Causes of mortality within the first 6 mo vs. after 6 mo

The top three known causes of mortality in the 41 patients who died within the first 6 mo were cardiovascular diseases (26.83%), respiratory failure (19.51%), and infectious diseases (9.76%) (Table 2). Furthermore, the top three causes of mortality after 6 mo were the same as those for mortality within the first 6 mo (Table 2).

Survival analysis for all-cause mortality according to NYHA classification

Considering that cardiovascular disease was the main reason for mortality within the first 6 mo and also after 6 mo, we further analyzed the survival of patients with different classes of cardiac function. As shown in Figure 3A, in the first 6 mo, the mortality rate for patients with baseline cardiac function of NYHA III-IV was much higher than that of patients without cardiac function limitation ($P = 0.003$). Similar results were found for these comparisons after 6 mo (Figure 3B).

Risk factors for mortality within the first 6 mo vs. after 6 mo

After correcting for confounding factors (serum calcium levels and blood glucose levels), multivariate Cox modeling analysis identified the following as independent risk factors for mortality within the first 6 mo in patients with diabetes receiving USPD: Increased age [hazard ratio (HR: 1.908; 95%CI: 1.400-2.600; $P < 0.001$]; lower levels of baseline serum creatinine (HR: 0.727; 95%CI: 0.614-0.860; $P < 0.001$); higher levels of baseline serum phosphorus (HR: 3.162; 95%CI: 1.848-5.409; $P < 0.001$); and NYHA class III-IV at baseline (HR: 2.148; 95%CI: 1.063-4.430; $P = 0.033$) (Figure 4A). Additionally, after adjusted serum creatinine calcium, phosphorus, and blood glucose levels, we found that advanced age (HR: 1.246; 95%CI: 1.033-1.504; $P = 0.022$) and baseline NYHA class III-IV (HR: 2.015; 95%CI: 1.298-3.130; $P = 0.002$) were risk factors for mortality after 6 mo (Figure 4B).

DISCUSSION

To the best of our knowledge, our study provides the first multicenter evaluation of the risk factors for mortality within the first 6 mo in patients with diabetes undergoing USPD. Advanced age and NYHA class III-IV at baseline were risk factors for mortality within the first 6 mo and after 6 mo; however, higher serum phosphorus levels and lower serum creatinine levels before dialysis were the only independent risk factors for mortality within the first 6 mo. The strength of the study was that it included data from five hospitals, making it representative and comprehensive.

Currently, the only report published on patients with diabetes undergoing USPD included 50 participants and reported an early mortality rate of 4.1%[14], similar to that of the present study. Moreover, we have demonstrated for the first time that the mortality in patients with diabetes undergoing USPD is highest in the first 6 mo. Thus, special attention should be paid to these patients

Table 1 Demographic and clinical characteristics of patients with diabetes undergoing urgent-start peritoneal dialysis

Index	Within the first 6 mo				After 6 mo			
	Died (*n* = 41)	Survived (*n* = 527)	χ^2/Z	*P*	Died (*n* = 113)	Survived (*n* = 395)	χ^2/Z	*P*
Demographic characteristics								
Age, yr, M (P_{25}, P_{75})	66.0 (57.0, 75.0)	58.0 (51.0, 66.0)	-4.003	< 0.001	61.0 (55.0, 67.0)	58.0 (50.0, 64.0)	-3.357	0.001
Gender, male, *n* (%)	22 (53.7)	322 (61.1)	0.882	0.348	65 (57.5)	244 (61.8)	0.666	0.414
Abdominal surgery history, *n* (%)	3 (7.3)	64 (12.1)	0.852	0.356	13 (11.5)	47 (11.9)	0.013	0.909
Co-morbidities, *n* (%)								
Cerebrovascular disease, *n* (%)	12 (29.3)	112 (21.3)	1.432	0.231	28 (24.8)	82 (20.8)	0.837	0.360
Hypertension, *n* (%)	40 (97.6)	512 (97.2)	0.000	1.000	110 (97.3)	384 (97.2)	0.006	0.941
NYHA-FC	-	-	11.647	0.009	-	-	9.265	0.026
0	14 (34.1)	254 (48.2)	-	-	46 (40.7)	196 (49.6)	-	-
I	4 (9.76)	97 (18.4)	-	-	16 (16.2)	79 (20.0)	-	-
II	4 (9.76)	55 (10.4)	-	-	14 (12.4)	39 (9.9)	-	-
III-IV	19 (46.3)	121 (23.0)	-	-	37 (32.7)	118 (23.2)		
Laboratory test								
WBC (10^9/L)	7.24 (6.11, 9.13)	7.26 (5.70, 8.60)	-0.674	0.501	6.82 (5.51, 7.75)	7.40 (5.77, 8.71)	-1.891	0.059
Alb (g/L)	31.20 (28.00, 33.95)	32.39 (28.80, 36.00)	-1.436	0.151	32.38 (29.00, 35.50)	32.30 (28.60, 36.00)	-0.202	0.840
Hb (g/L)	86.0 (74.5, 98.0)	86.0 (74.0, 97.0)	-0.214	0.831	85.0 (74.0, 95.0)	86.0 (74.0, 97.0)	-0.677	0.498
K (mmol/L)	4.57 (3.91, 5.11)	4.57 (4.00, 5.09)	-0.195	0.845	4.50 (3.95, 4.92)	4.61 (4.01, 5.13)	-1.442	0.149
Na (mmol/L)	140.00 (136.60, 142.50)	140.03 (138.00, 142.30)	-0.909	0.363	140.10 (137.95, 142.85)	140.10 (138.00, 142.30)	-0.364	0.176
Ca (mmol/L)	1.95 (1.77, 2.13)	1.97 (1.84, 2.11)	-0.875	0.382	1.99 (1.88, 2.11)	1.96 (1.81, 2.11)	-1.348	0.178
P (mmol/L)	2.00 (1.60, 2.41)	1.87 (1.48, 2.16)	-1.145	0.252	1.71 (1.42, 2.05)	1.88 (1.53, 2.18)	-3.242	0.001
Boold glucose (mmol/L)	6.91 (5.60, 9.96)	6.16 (4.83, 7.70)	-2.555	0.011	6.30 (4.87, 8.52)	6.16 (4.83, 7.56)	-0.547	0.584
Scr (μmol/L)	646.80 (484.45, 806.85)	710.00 (562.00, 876.10)	-1.855	0.064	642.00 (532.25, 840.25)	723.05 (578.80, 887.80)	-2.626	0.009

NYHA-FC: New York Heart Association functional classification.; WBC: White cell count; Hb: Hemoglobin; Alb: Albumin; Scr: Serum creatinine; Na: Sodium; K: Potassium; Ca: Calcium; P: Phosphorus.

during this time period. As in previous studies[19,20], the leading cause of mortality within the first 6 mo for the USPD recipients with diabetes in our study was cardiovascular disease. Active treatment is therefore required at an early stage to reduce the risk of mortality due to cardiovascular events in these patients.

In agreement with previous reports[4,5,21], we identified advanced age as an independent risk factor not only for mortality within the first 6 mo but also after 6 mo of USPD in patients with diabetes. A reasonable explanation is that advanced age increases the incidence of cardiovascular events and consequently mortality in patients with diabetes[22]. Immune dysfunction and microinflammation in patients with renal failure can easily lead to sepsis, which increases the risk of mortality. The more advanced the age, the worse the immune function and, consequently, the greater the risk of mortality [23].

We found that the baseline serum creatinine level inversely correlated with mortality within the first 6 mo of USPD in patients with diabetes. We believe that the condition of the patient mainly accounts for this linkage. Patients with diabetes who start dialysis urgently often present with severe symptoms rather than biochemical indicators of severe renal failure. A lower serum creatinine level in patients with diabetes before USPD is reflective of an earlier initiation of emergency peritoneal dialysis, which contrarily reflects more severe symptoms in the patient at initial presentation. However, the baseline serum creatinine level was not an independent risk factor for mortality after 6 mo. A possible reason was that as the course of USPD progressed, the patient's condition improved, and the baseline serum

Table 2 Reasons for mortality in patients with diabetes undergoing urgent-start peritoneal dialysis during different follow-up periods

Causes	Number of mortalities within the first 6 mo, *n* (%)	Number of the mortalities after 6 mo, *n* (%)
Infectious diseases	4 (9.76)	16 (14.16)
Cardiovascular events	11 (26.83)	36 (31.86)
Cerebrovascular disorder	3 (7.32)	10 (8.85)
Respiratory failure	8 (19.51)	14 (12.39)
Malignancy	3 (7.32)	3 (2.66)
Multiple organ dysfunction	3 (7.32)	2 (1.77)
Unknown	9 (21.95)	32 (28.32)
Total	41 (100.00)	113 (100.00)

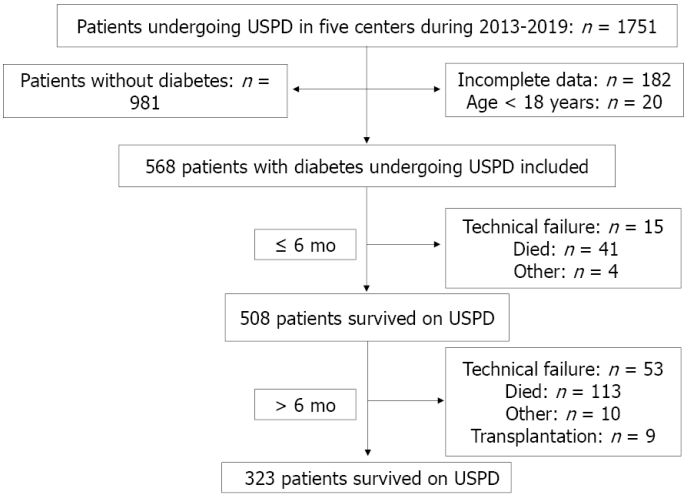

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Figure 1 Patient distribution over two follow-up periods. USPD: Urgent-start peritoneal dialysis.

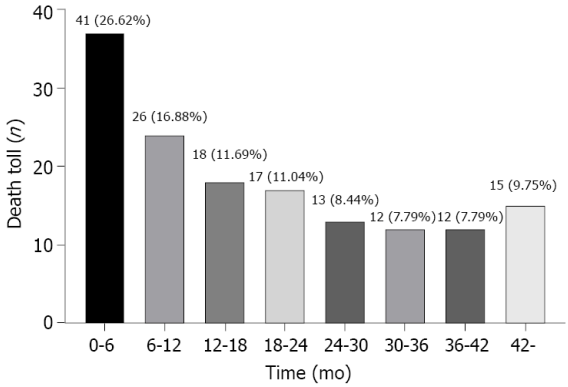

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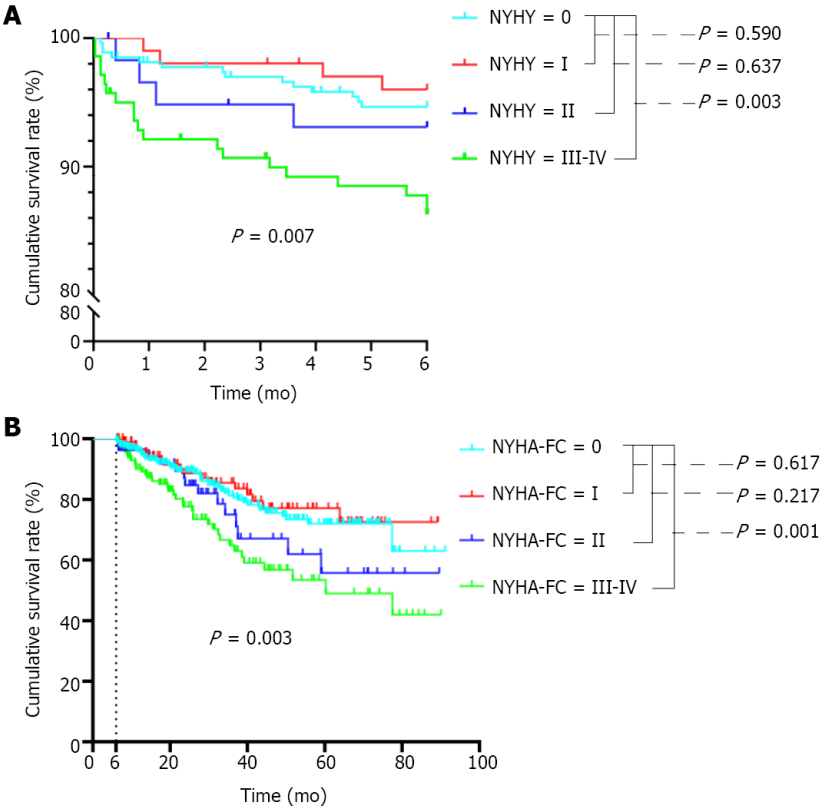
Figure 2 Mortality rates for patients with diabetes undergoing urgent-start peritoneal dialysis during the follow-up period. The percent indicates the proportion of mortalities in each period to the mortalities during the entire follow-up period.

creatinine level did not reflect the disease severity on follow-up; therefore, the results indicated that USPD alleviated the patient's condition.

For patients with diabetes undergoing USPD, a link between the serum phosphorus level and mortality has not been reported. For these patients, we found that the risk of mortality within the first 6 mo increased by 216.2%% for each 1 mmol/L increase in the baseline serum phosphorus level. Other studies have shown that a high level of serum phosphate correlates with vascular calcification in uremic



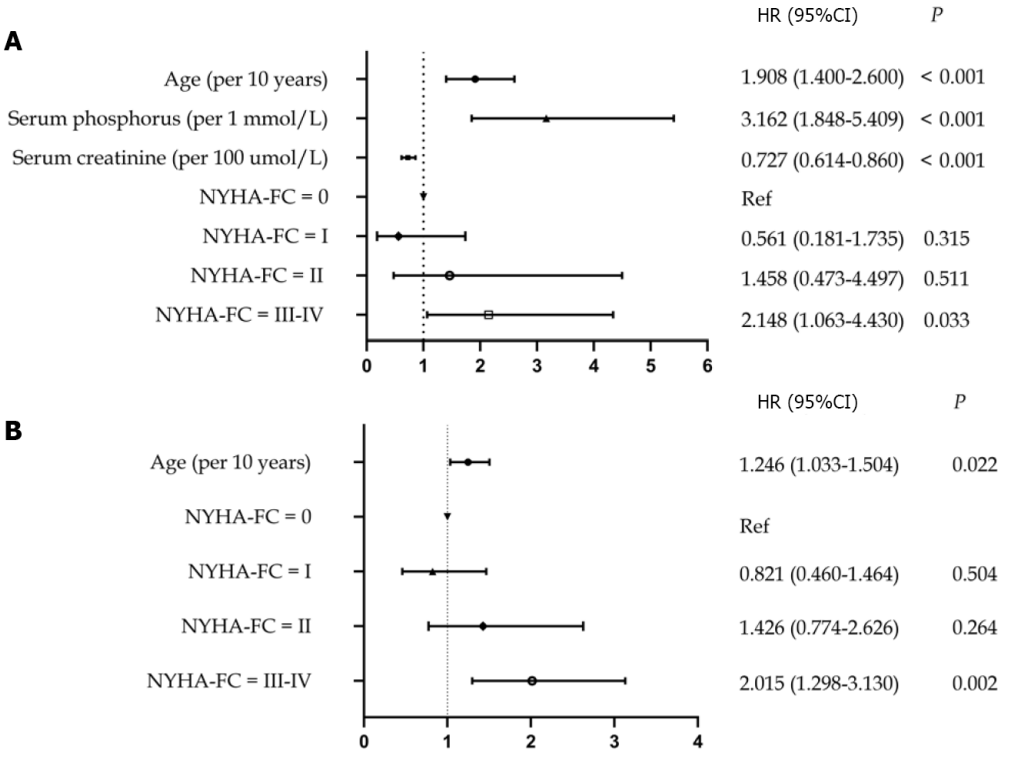
DOI: 10.4239/wjd.v13.i4.376

Figure 3 Kaplan-Meier survival analysis for all-cause mortality according to New York Heart Association functional classification. A: Kaplan-Meier survival analysis for all-cause mortality according to New York Heart Association functional classification (NYHA-FC) within the first 6 mo; B: Kaplan-Meier survival analysis for all-cause mortality according to NYHA-FC after 6 mo. NYHA-FC: New York Heart Association functional classification.

patients[24-26], and that vascular calcification increases the risk of myocardial infarction[27], coronary artery disease[28], and mortality[29]. Serum phosphate level has also been found to be a powerful independent predictor of coronary heart disease in patients with diabetes[28]. Baseline serum phosphorus level was not a risk factor for mortality after 6 mo, which is similar to findings in other studies[30,31]. In our study, we focused on the risk factors for mortality within the first 6 mo; therefore, we only collected the baseline serum phosphorus levels. We speculated that with the progress of dialysis and the use of phosphorus-reducing drugs during dialysis, serum phosphorus levels would gradually be corrected; therefore, the baseline serum phosphorus level is not reflective of the overall level after 6 mo of treatment. This suggests that it is crucial and beneficial to control the serum phosphorus level of patients in the initial stage of dialysis to reduce early mortality.

Patients with ESRD and diabetes are more likely to develop cardiovascular diseases than are non-diabetic patients with ESRD[32]. Additionally, it was proved that patients on dialysis with poor cardiac function have a very poor prognosis[33]. Therefore, exploration of the relationship between mortality and cardiac function in patients with diabetes undergoing USPD is critical and significant. We found that among patients with diabetes undergoing USPD, those with obvious heart failure symptoms have a higher risk of mortality within the first 6 mo and after 6 mo than do those with mild or no heart failure symptoms. We found that NYHA class III-IV was a risk factor for mortality both within and after the first 6 mo; however, the risk of mortality after 6 mo was lower than that within the first 6 mo for patients with poorer baseline cardiac function. As a possible cause, we speculated that patients with poorer baseline cardiac function are more likely to suffer from complications of heart disease, and after a series of treatments such as dialysis, although their cardiac function improves, it cannot be completely corrected; therefore, the risk of mortality is merely reduced. Thus, routine monitoring of the patient's cardiac function in the early stages of dialysis is advised. Moreover, when the patient's cardiac function is not ideal, appropriate measures should be taken to promptly improve it.

Our study had several limitations. First, because it was retrospective, information bias could not be avoided. For example, the laboratory indicators such as cardiac function and serum phosphorus levels at different time points had not been determined, and the cause of mortality could not always be precisely established. Second, our sample size was small, and larger studies are needed to accurately predict mortality within the first 6 mo in diabetic patients undergoing USPD to provide further guidance for clinical applications.



DOI: 10.4239/wjd.v13.i4.376

Figure 4 Risk factors for mortality in patients with diabetes undergoing urgent-start peritoneal dialysis during different periods. A Cox proportional hazard regression model was used to identify the risk factors for mortality during different periods of follow-up. A: Risk factors for mortality in patients with diabetes undergoing USPD within the first 6 mo; B: Risk factors for mortality in patients with diabetes undergoing USPD after 6 mo. NYHA-FC: New York Heart Association functional classification; USPD: urgent-start peritoneal dialysis. HR: Hazard ratio; CI: Confidence interval.

CONCLUSION

The risk of mortality within the first 6 mo in patients with diabetes was the highest after USPD initiation. We suggest that controlling serum phosphorus levels and improving cardiac function will decrease the risk of mortality within the first 6 mo in these patients.

ARTICLE HIGHLIGHTS

Research background

Many patients with end-stage renal disease have to choose urgent-start peritoneal dialysis (USPD), and patients with diabetes mellitus (DM) who are undergoing USPD have a poorer prognosis than do those without DM. The first 6 mo after the start of urgent dialysis is a high-risk period, and for patients with DM undergoing USPD, we speculate that the mortality risk is high in the first 6 mo after USPD. However, the distribution of mortalities over time and the risk factors for mortality within the first 6 mo in this patient population has not been reported. Thus, it is important to identify the risk factors for mortality within the first 6 mo of USPD initiation in patients with DM.

Research motivation

We hoped to identify the reasons for the poor prognosis of patients with DM undergoing USPD.

Research objectives

The main aim of this study was to identify risk factors for mortality within the first 6 mo in patients with DM undergoing USPD in order to facilitate better management of such patients in clinical practice.

Research methods

In this multicenter, retrospective cohort study, we screened patients with ESRD who underwent USPD at five hospitals. To highlight the specificity of risk factors within the first 6 mo, we divided the follow-up period into two survival phases: the first 6 mo and the months thereafter. We compared the survival rates of patients with different cardiac function classes in each period using Kaplan-Meier curves. The

risk factors for mortality during the different periods were analyzed using a Cox proportional hazard regression model.

Research results

We found that the highest proportion (26.62%) of mortalities occurred between 0 and 6 mo. The mortality rate for patients with baseline cardiac function represented by New York Heart Association (NYHA) III-IV was much higher than that for patients without cardiac function limitation, both within the first 6 mo and after 6 mo (all $P < 0.05$). Increased age ($P < 0.001$), lower levels of baseline serum creatinine ($P < 0.001$), higher levels of baseline serum phosphorus ($P < 0.001$), and NYHA class III-IV at baseline ($P = 0.033$) were risk factors for mortality within the first 6 mo. The risk factors for mortality after 6 were advanced age ($P = 0.022$) and baseline NYHA class III-IV ($P = 0.002$).

Research conclusions

This study suggests the importance of controlling serum phosphorus levels and improving cardiac function for decreasing the mortality risk within the first 6 mo in patients with DM undergoing USPD.

Research perspectives

Further research is needed to build a model to predict the risk of mortality within the first 6 mo in patients with DM undergoing USPD.

ACKNOWLEDGEMENTS

We thank all the staff of the five peritoneal dialysis for providing data, which made this paper possible.

FOOTNOTES

Author contributions: All the authors solely contributed to this paper; Cheng SY analyzed the data and wrote this manuscript; Yang LM, Sun ZS, Zhang XX, and Zhu XY provided the data; Meng LF, Guo SZ, and Zhuang XH collected the data; Luo P organized the study; Cui WP designed this study and reviewed this manuscript.

Supported by Jilin Province Health and Technology Innovation Development Program Funded Project, No. 2018FP031.

Institutional review board statement: This retrospective study was approved by the Ethics Committee of the Second Hospital of Jilin University (design number, 2020031).

Informed consent statement: The requirement for informed consent was waived owing to the retrospective nature of the study.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at wenpengcui@163.com

STROBE statement: The guidelines of the STROBE statement have been adopted for this study.

Country/Territory of origin: China

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S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 May 15; 13(5): 387-421

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yu-Xi Chen*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Jia-Ping Yan*.

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Manfredi Rizzo, Michael Horowitz

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World J Diabetes 2022 May 15; 13(5): 387-407

DOI: 10.4239/wjd.v13.i5.387

ISSN 1948-9358 (online)

FRONTIER

Role of cannabinoids and the endocannabinoid system in modulation of diabetic cardiomyopathy

Mona F El-Azab, Ahmed E Wakiel, Yossef K Nafea, Mahmoud E Youssef

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Koustas E, Greece; Wang CY, Taiwan; Zhang ZL, China

Received: May 31, 2021
Peer-review started: May 31, 2021
First decision: July 3, 2021
Revised: July 18, 2021
Accepted: April 28, 2022
Article in press: April 28, 2022
Published online: May 15, 2022

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Abstract

Diabetic complications, chiefly seen in long-term situations, are persistently deleterious to a large extent, requiring multi-factorial risk reduction strategies beyond glycemic control. Diabetic cardiomyopathy is one of the most common deleterious diabetic complications, being the leading cause of mortality among diabetic patients. The mechanisms of diabetic cardiomyopathy are multi-factorial, involving increased oxidative stress, accumulation of advanced glycation end products (AGEs), activation of various pro-inflammatory and cell death signaling pathways, and changes in the composition of extracellular matrix with enhanced cardiac fibrosis. The novel lipid signaling system, the endocannabinoid system, has been implicated in the pathogenesis of diabetes and its complications through its two main receptors: Cannabinoid receptor type 1 and cannabinoid receptor type 2, alongside other components. However, the role of the endocannabinoid system in diabetic cardiomyopathy has not been fully investigated. This review aims to elucidate the possible mechanisms through which cannabinoids and the endocannabinoid system could interact with the pathogenesis and the development of diabetic cardiomyopathy. These mechanisms include oxidative/nitrative stress, inflammation, accumulation of AGEs, cardiac remodeling, and autophagy. A better understanding of the role of cannabinoids and the endocannabinoid system in diabetic cardiomyopathy may provide novel strategies to manipulate such a serious diabetic complication.

Key Words: Δ 9-tetrahydrocannabinol; Autophagy; Cannabinoid receptors; Diabetic cardiomyopathy; Endocannabinoid system; Inflammation

Core Tip: Diabetic cardiomyopathy is considered to be one of the most common deleterious diabetic complications being the leading cause of mortality among diabetic patients. The endocannabinoid system has been implicated in the pathogenesis of diabetes and its complications. However, the role of the endocannabinoid system in diabetic cardiomyopathy has not been fully investigated. This review aims to elucidate the possible mechanisms through which cannabinoids and the endocannabinoid system could interact with the pathogenesis of diabetic cardiomyopathy. Better understanding of the role of cannabinoids and the endocannabinoid system in diabetic cardiomyopathy may provide novel strategies to manipulate this serious diabetic complication.

Citation: El-Azab MF, Wakiel AE, Nafea YK, Youssef ME. Role of cannabinoids and the endocannabinoid system in modulation of diabetic cardiomyopathy. *World J Diabetes* 2022; 13(5): 387-407
URL: https://www.wjgnet.com/1948-9358/full/v13/i5/387.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i5.387

INTRODUCTION

Diabetes mellitus is one of the most common chronic disorders worldwide, and it continues to increase in number and significance. The total number of individuals with diabetes worldwide is 463 million, with a prevalence rate of 9.3% according to the International Diabetes Federation[1]. It is estimated that the prevalence of diabetes on a global scale could reach 578 million by 2030 and 700 million by 2045. In 2017, diabetes-related mortality accounted for 4 million people worldwide and the total healthcare expenditure reached 727 billion United States Dollars[2].

Diabetes mellitus is a complex metabolic condition that is characterized by hyperglycemia resulting from a lack of absolute or relative insulin[3]. It is linked to insulin resistance in many instances. Type 1 diabetes is caused by an autoimmune destruction of insulin-secreting cells in the pancreas, and type 2 diabetes is caused by insufficient compensatory insulin production in the presence of peripheral insulin resistance. Ninety percent of diabetes cases are of the latter type[4].

Both microvascular (retinopathy, nephropathy, and neuropathy)[5-9] and macrovascular (cardiovascular disease) problems are linked to diabetes[6-10]. Despite substantial advances in anti-diabetic therapy, diabetic complications, which are most commonly recognized in the long-term, are consistently harmful to a large extent, necessitating multi-factorial risk reduction measures beyond glycemic control[11]. Diabetes-related morbidity and mortality are primarily caused by cardiovascular problems[12]. Indeed, 50% of diabetic patients die of a cardiovascular disease[13]. Endothelial dysfunction, coronary artery disease, and myocardial left ventricular dysfunction (which leads to heart failure) are all well-known cardiovascular problems[14]. Diabetic patients have a 2-4 times higher risk of heart failure than non-diabetic patients, according to clinical research[15,16].

Diabetic cardiomyopathy is a deficiency in ventricular contractile function that occurs in diabetic individuals regardless of the presence of coronary artery disease or other cardiovascular disorders. It is a complicated diabetes-related condition marked by severe alterations in the heart's physiology, anatomy, and mechanical performance[17]. Diabetic cardiomyopathy is a complicated and poorly understood process. To explain the structural and functional alterations associated with diabetic cardiomyopathy, several pathogenic processes have been explored and suggested[18]. Increased oxidative/nitrative stress[19-21], accumulation of advanced glycation end products (AGEs)[22], activation of various pro-inflammatory and cell death signaling pathways[23], and changes in the composition of extracellular matrix with elevated cardiac fibrosis[24] are some of the proposed pathological mechanisms. Unfortunately, despite the growing body of information concerning diabetic cardiomyopathy over the last few decades, therapeutic choices remain inadequate. Other treatments for diabetic cardiomyopathy's multi-factorial pathogenic pathways have yet to be developed.

The endocannabinoid system is an endogenous lipid signaling system that consists of: (1) Two main receptors identified as cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2); (2) Endogenous ligands for these two receptors known as endocannabinoids; and (3) Proteins that control endocannabinoid tissue levels (anabolic and catabolic enzymes)[25]. The endocannabinoid system has become a novel therapeutic target in a range of cardiovascular illnesses in the last decade, including atherosclerosis, myocardial infarction, and heart failure[26]. Furthermore, the significance of the endocannabinoid system in the development of diabetes and associated consequences has been suggested in various pre-clinical and clinical research[27-29]. The possible mechanisms through which cannabinoids and the endocannabinoid system could modulate the pathogenesis of diabetic cardiomyopathy are highlighted in this review (Table 1), an approach that could pave the way for the use of this

Table 1 Role of cannabinoid agents in diabetes

Cannabinoid agent	Mechanism	Role in diabetes
Anandamide	Endogenous cannabinoid	Elevated in diabetic patients[26]
	CB1 agonist	
	CB2 agonist	
Rimonabant (SR141716A)	CB1 antagonist	Reduced weight[62]
		Reduced hemoglobin A1c levels[62]
		Reduced fasting blood glucose levels[62]
		Reduced high density lipoprotein, cholesterol and triglyceride levels[62]
		Improved systolic blood pressure[62]
∆ 9-tetrahydrocannabinol (THC)	Psychoactive cannabinoid	Lowered blood glucose level[65]; Preserved pancreatic insulin content[65]
	CB1 partial agonist	
	CB2 partial agonist	
Cannabidiol	Non-psychoactive cannabinoid	Reduced the incidence of type I diabetes[66]
	Low affinity to CB1 and CB2	Immunosuppressive effect[66]

system as an effective tool in the management of these harmful diabetic complications.

DIABETIC CARDIOMYOPATHY: A DISTINCT COMPLEX DISORDER

Cardiomyopathies are a group of diseases characterized by myocardial dysfunction that is not induced by common causes, such as coronary artery disease, valvular dysfunction, or hypertension. Cardiomyopathies are divided into four categories depending on hemodynamic characteristics: Dilated, hypertrophic, restrictive, and obliterative cardiomyopathy[30]. Dilated cardiomyopathy is characterized by ventricular dilatation and systolic dysfunction, which commonly affects both ventricles. The most common symptom of hypertrophic cardiomyopathy is significant ventricular hypertrophy. Restrictive cardiomyopathy is characterized by inflexible and poorly distensible myocardium, resulting in poor compliance. Endo-myocardial fibrosis is a symptom of obliterative cardiomyopathy. The endocardium's severe fibrosis encroaches on and reduces the size of the ventricular cavities[31]. Diabetic cardiomyopathy can be classified as either dilated or hypertrophied cardiomyopathy[32].

Rubler *et al*[33] coined the name diabetic cardiomyopathy in 1972 after observing a specific type of cardiomyopathy in diabetic patients who did not have other cardiovascular issues such as coronary artery disease, valvular or congenital heart disease, or hypertension. Diabetic cardiomyopathy is defined by a series of cardiac alterations, including interstitial fibrosis, myocardial hypertrophy, and microcirculatory abnormalities, that arise due to diabetes mellitus. These circulatory issues impair heart function, eventually leading to cardiac failure[4]. Heart failure lowers an individual's quality of life and makes diabetes control more difficult. As a result, early diagnosis and treatment of these patients are regarded as top priorities[34].

Insulin resistance and hyperglycemia are significant drivers in diabetic patients, activating a variety of adaptive and maladaptive responses that ultimately affect cardiac function[35]. To explain the complicated structural and functional abnormalities associated with diabetic cardiomyopathy, several pathogenic processes have been examined and proposed (Figure 1). These systems work in concert and may even enhance one another[36]. Hyperglycemia increases oxidative stress by accelerating glucose oxidation and mitochondrial production of reactive oxygen species (ROS), which induce DNA damage and promote apoptosis[37]. AGEs build up in tissues, including the myocardium, and have been linked to structural abnormalities in diabetic hearts[22]. Activation of numerous pro-inflammatory and stress signaling pathways, such as mitogen activated protein kinases (MAPKs), also stimulates apoptotic pathways and cell death, and promote myocardial cell death[23,38]. Finally, there is increased collagen formation in the myocardium that leads to fibrosis and reduced contractile function of the heart[24,39].

Diabetic cardiomyopathy is divided into three stages (Figure 2): Early-stage, middle-stage, and late-stage[4]. In the early stage, the heart develops hypertrophy and has diastolic dysfunction with normal ejection fraction, and it is asymptomatic[40]. Increased left ventricular size, wall thickness, and mass, as well as diastolic dysfunction and a modest decline in systolic performance, characterize the intermediate stage. Insulin resistance, AGE formation, elevated renin-angiotensin-aldosterone system levels, apoptosis, necrosis, and fibrosis are all associated with this stage[41]. As the disease progresses from the

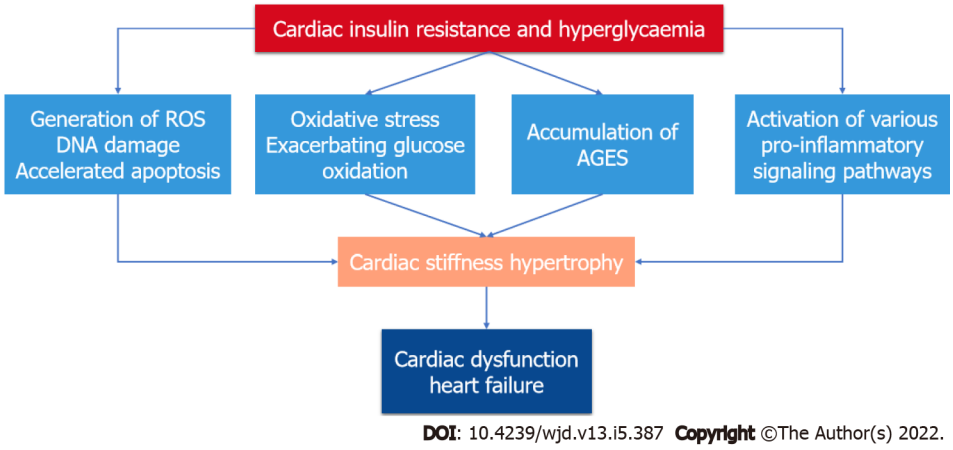


Figure 1 Molecular mechanisms of diabetic cardiomyopathy. Hyperglycemia and insulin resistance increase reactive oxygen species formation, oxidative stress, advanced glycation end-products formation, and the recruitment of various inflammatory pathways leading to cardiac dysfunction and heart failure. ROS: Reactive oxygen species.

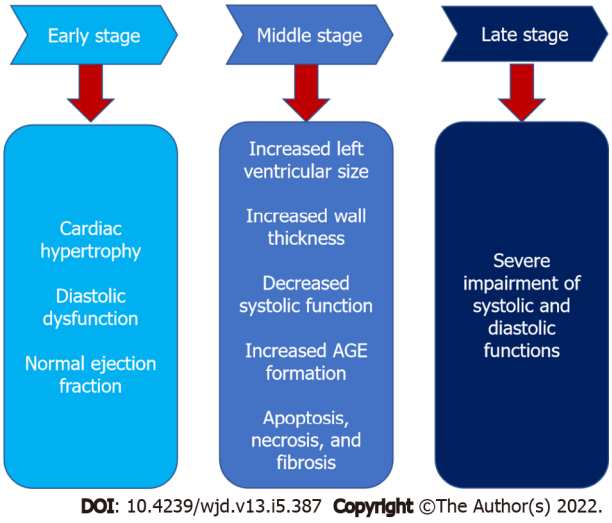


Figure 2 Stages of diabetic cardiomyopathy progression. Diabetic cardiomyopathy progresses from early development of hypertrophy and diastolic dysfunction that then progress to decreased systolic activity, apoptosis and cardiac fibrosis leading to severe impairment in both systolic and diastolic functions. AGE: Advanced glycation end products.

medium to late stage, it becomes more severe, impairing both systolic and diastolic functioning[13].

THE ENDOCANNABINOID SYSTEM

The discovery of the endogenous signaling system now recognized as the endocannabinoid system began with the chemical detection of 9-tetrahydrocannabinol (THC), the main psychoactive component of *Cannabis sativa*[42]. THC's psychotropic and immunomodulatory effects are due to the ability to bind to and activate specific receptors, including the CB1 receptor, which is one of the most abundant G-protein-coupled receptors in the central nervous system[43], and the CB2 receptor, which is abundantly expressed in several immune cells and tissues[44].

The existence of endogenous substances (the endocannabinoids) capable of binding to and activating CB1 and CB2 receptors was suggested. Anandamide (N-arachidonoyl ethanolamine)[45] and 2-arachidonoyl glycerol (2-AG)[46] are the two most well-studied examples of these compounds. The endocannabinoid system is made up of cannabinoid receptors, endocannabinoids, and proteins that catalyze endocannabinoid biosynthesis (N-acyl-phosphatidylethanolamine phospholipase-D for anandamide and diacylglycerol lipases for 2-AG), transport, and inactivation [fatty acid amide hydrolase (FAAH) for anandamide and monoacyl glycerol lipase for 2-AG][47]. Signaling *via* CB1 and CB2 receptors is complex, involving inhibition (and activation in some cases) of adenyl cyclase activity,

activation of various MAPKs [*e.g.*, p38- and p44/42-MAPKs, c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinase (ERK)], protein kinases A and C (PKA and PKC), and modulation of various calcium and potassium channels[48].

Since its discovery about two decades ago, the endocannabinoid system has gained considerable importance as a fundamental signaling system implicated in almost all physiological and pathological processes in animals[49]. In a wide range of pathological conditions, including mood and anxiety disorders, movement disorders, neuropathic pain, multiple sclerosis, cancer, glaucoma, osteoporosis, reproductive disorders, immune dysfunction, cardiovascular and metabolic disorders, there is growing evidence that the endocannabinoid system plays pivotal roles and holds tremendous therapeutic options[50,51].

Besides the primary distribution of CB1 receptors in the CNS and CB2 receptors in immune cells, as these are responsible for the psychoactive and immunomodulatory effects of cannabinoids, both receptors have been found to be expressed in cardiovascular system cells such as cardiomyocytes, fibroblasts, endothelial and vascular smooth muscle cells, and infiltrating immune cells[26]. CB1 receptors activation by endocannabinoids or synthetic ligands has complex depressive effects in the cardiovascular system and has been linked to the development of pathophysiological alterations and compromised cardiovascular function in various forms of shock[51] and heart failure[52]. In addition, several studies indicated that stimulation of CB1 receptors in the cells of the cardiovascular system is associated with activation of stress signaling pathways promoting cell death, ROS production, and induction of inflammatory cascades[26,52]. On the other hand, an increased CB2 receptor expression has been reported in the cardiovascular system under pathophysiological conditions such as inflammatory stimulation or tissue injury, which likely reflects a protective response to limit these effects[53]. A great body of evidence suggest a protective role of CB2 receptors in experimental models of cardiovascular disorders including mouse models of atherosclerosis[54], restenosis[55] and myocardial ischemia/reperfusion injury[56].

Different expression patterns of CB1 and CB2 receptors together with other components of the endocannabinoid system such as synthesizing and degrading enzymes have been reported in islet cells of humans, rats and mice[57-59]. There are controversial results regarding the role of CB1 receptors in insulin secretion with studies showing an increased insulin secretion in islet cells by activation of CB1 receptors[57,58], and others showing decreased insulin secretion[60]. In addition, activation of CB2 receptors in islet cells has also been shown to either stimulate[61] or attenuate insulin secretion[57]. Several studies have found that the endocannabinoid system plays a significant role in the etiology of diabetes. Serum levels of anandamide and 2-AG have been found to be greater in type 2 diabetics than in healthy individuals[27]. Furthermore, in these diabetic patients, subcutaneous tissue levels of anandamide were found to be elevated, indicating endocannabinoid system overactivity[28].

A clinical trial was conducted in obese patients with type 2 diabetes inadequately controlled by either metformin or sulfonylureas using the CB1 antagonist rimonabant (SR141716A). Rimonabant treatment caused a reduction in weight, hemoglobin A1c levels, fasting blood glucose, high-density lipoprotein cholesterol and triglycerides, as well as improvement in systolic blood pressure[62]. In the type 2 diabetic patients naive to anti-diabetic treatment, rimonabant showed similar results with improved glycemic control and metabolic profile[63]. Another study demonstrated that the treatment of type 2 diabetic patients on standard insulin treatment with rimonabant also improved glycemic control and the metabolic profile[64]. The psychoactive cannabinoid THC was shown to attenuate the severity of autoimmune responses in an experimental model of autoimmune diabetes in addition to lowering blood glucose level and preserving pancreatic insulin content[65]. Unfortunately, the psychoactive effects of THC hampered this therapeutic approach. The non-psychoactive cannabidiol (CBD) reduced the incidence of diabetes in a mouse model of type 1 diabetes, an effect that involved immunosuppressive and anti-inflammatory effects[66].

The majority of diabetic complications are linked to abnormalities in the vascular system[67]. Hyperglycemia has been related to a number of critical processes, including oxidative/nitrative damage, AGE buildup, and inflammatory system stimulation[68]. Endothelial dysfunction occurs in arteries, which contributes to the development of numerous diabetes problems. Indeed, cannabinoids and the endocannabinoid system represent an outstanding therapeutic approach to manage these deleterious complications. Interestingly, this notion is supported by a great body of evidence implicating the endocannabinoid system in the pathogenesis of nearly all diabetic complications including nephropathy, retinopathy, and neuropathy, in addition to cardiovascular complications, mainly through modulation of the aforementioned mechanisms[29]. Still, the role of the endocannabinoid system in diabetic cardiomyopathy; the distinct diabetic complication, has not been fully investigated in detail.

POSSIBLE MECHANISMS THROUGH WHICH CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM COULD MODULATE DIABETIC CARDIOMYOPATHY

Oxidative/Nitrative stress

Nearly 95% of oxygen consumed by tissues is used in metabolic processes to produce adenosine triphosphate (ATP), and approximately 5% of oxygen consumed is transformed into superoxide (O^{2-}) radical, the principal oxygen free radical produced by mitochondria[69]. The antioxidant enzymes superoxide dismutase (SOD1, SOD2, and SOD3) quickly convert superoxide to hydrogen peroxide (H2O2) within the cell[70]. Antioxidant enzymes such as catalase, glutathione peroxidase, and other peroxidases generally convert excess H2O2 to harmless water[71]. Although H2O2 is not a free radical, it can undergo the Fenton reaction with reduced transition metals [*e.g.*, ferrous ion (Fe^{2+})] or with superoxide in the presence of metal ions (usually iron or copper) to produce the highly reactive hydroxyl radical (OH), which is a far more damaging molecule to the cell[72]. Superoxide radicals can quickly react with nitric oxide (NO) to produce cytotoxic peroxynitrite anions (ONOO–) in addition to producing H2O2[73]. Superoxide and NO are less reactive than peroxynitrite, which might combine with carbon dioxide to generate nitrotyrosine, that triggers protein degradation and lipid oxidation[74].

Besides mitochondria, other cellular sources of reactive oxygen and nitrogen species (RNS) exist. NADPH oxidase, for example, promotes the enzymatic conversion of oxygen to superoxide anion. Several critical cytosolic proteins (p44phox, p67phox, p40phox, and Rac2) must be translocated to the cellular membrane for NADPH oxidase activation[75]. Other sources of ROS and RNS, in addition to NADPH oxidase, include nitric oxide synthase (NOS), which stimulates NO synthesis[76], and peroxisomes, that are known to create H2O2 primarily through fatty acid oxidation[77] and phagocytic cell activation[78].

The oxidative stress pathway has emerged as a common thread connecting all major diabetic cardiomyopathy pathophysiological mechanisms[79]. These pathways are the result of a single hyperglycemia-induced process: The overproduction of superoxide by the mitochondrial electron transport chain[80]. Formation of AGE products, auto-oxidation of glucose, activation of PKC, and NADPH oxidase are some of the other sources of ROS in diabetes[81]. Once oxidative stress develops, it results in a vicious self-sustaining cycle of generating more free radicals and causing more stress as a result of the activation of multiple stress-induced pathways and due to its ability to cause damaging effects to multiple components within the cell[82].

Through a variety of mechanisms, ROS induce cellular damage in the diabetic myocardium. Increased ROS directly damage cellular proteins and DNA[83]. In addition, ROS activate matrix metalloproteinases, which modify the extracellular matrix architecture and cause fibrosis[84], as well as regulating signal transduction pathways that cause cardiomyocyte hypertrophy[85] and apoptosis, which results in the loss of contractile tissue[86]. In a similar manner, peroxynitrite induces vasoconstriction, enhanced leukocyte adherence, platelet activation, oxidation, pro-thrombotic state, impaired coagulation, and vascular inflammation, among other pro-atherosclerotic pathogenic processes[87]. In type 1 diabetic mice, selective suppression of mitochondrial ROS was demonstrated to prevent diabetic cardiac abnormalities, confirming the importance of mitochondrial ROS role in developing cardiac abnormalities[88]. Moreover, Rac1 increases mitochondrial ROS generation *via* NADPH oxidase activation and plays an important role in cardiomyocyte death and cardiac failure in streptozotocin-induced diabetes in mice[89].

Previous studies have shown that the endocannabinoid system can influence ROS and RNS production, implying that modulating the endocannabinoid system and administering exogenous cannabinoids with antioxidant properties could be beneficial in the treatment of diabetes-related cardiovascular complications, such as diabetic cardiomyopathy[29].

It has been shown that genetic deletion of CB1 receptors attenuated the rise in markers of oxidative [4-hydroxy-trans-2-nonenal (4-HNE)] and nitrative (nitrotyrosine) stress in the myocardium of mice treated with acute or chronic doses of the potent, cardio-toxicant, anticancer drug doxorubicin[90]. In addition, doxorubicin treatment led to decreased myocardial content of the components of the antioxidant defense system: Glutathione, glutathione peroxidase, and SOD. These changes were significantly reduced in the myocardium of CB1 knockout mice[90]. Consistent with the data obtained from rodents, activation of CB1 receptors by anandamide or the potent agonist HU210, with or without doxorubicin, induced ROS production in human primary cardiomyocytes (HCM). The previous deleterious effect was attenuated by the use of CB1 antagonists: SR141716A or AM281[90].

Mukhopadhyay *et al*[52] similarly found that pharmacological blockage of CB1 receptors with AM281 or SR141716A reduced doxorubicin-induced oxidative/nitrative stress and related cell death. In comparison to their wild-type counterparts, mice lacking the FAAH gene showed a significant increase in acute and chronic doxorubicin-induced cardiac oxidative and nitrative stress, as well as impaired antioxidant defense and tissue injury[91]. Furthermore, anandamide increased the sensitivity of inflammatory cells isolated from FAAH mutant mice to ROS generation. These findings imply that, in pathological situations involving oxidative/nitrative stress (such as doxorubicin-induced myocardial injury), FAAH plays an important role in regulating endocannabinoid-induced cardiac cell injury, which is mediated in part by CB1 receptor activation because these effects may be attenuated by

selective CB1 antagonists[91].

The role of the endocannabinoid system in oxidative stress control has also been proven in atherosclerosis models such as the apolipoprotein E (ApoE) deficient animal model. In ApoE and CB2 double knockout mice, the release of superoxide radical was increased two-fold in intact aortic segments compared to ApoE knockout mice. The selective CB2 agonist JWH-133 reduced ROS release in ApoE knockout mice to comparable levels to those in wild-type animals[54].

The first evidence of a direct link between the endocannabinoid system and the pathogenesis of diabetic cardiomyopathy came from the interesting study conducted by Rajesh and co-workers in 2011. This research group demonstrated an increased expression of CB1 receptors and anandamide levels in the myocardium of streptozotocin-induced diabetic mice compared to their non-diabetic counterparts [92]. Streptozotocin-induced diabetic cardiomyopathy was characterized by a profound accumulation of markers of oxidative and nitrative stress in the myocardium, an effect that was ameliorated by genetic deletion of CB1 receptors. In addition, genetic deletion of CB1 mitigated the expression of the $p40^{phox}$ NADPH oxidase active subunit in myocardial tissue of diabetic mice[92].

Earlier, the same research group demonstrated a protective effect of CBD in diabetic cardiomyopathy [92]. CBD is the most common non-psychotropic cannabinoid in *Cannabis sativa*, and it has been approved for the treatment of inflammation, pain, and spasms associated with multiple sclerosis in humans[93]. CBD exerts several actions that are independent of the CB1 and CB2 receptors[94]. In this study, CBD therapy was found to reduce oxidative and nitrative stress in the myocardium of streptozotocin-induced diabetic mice. Additionally, CBD was found to reduce ROS production as well as the expression of active ROS-generating NADPH oxidase isoforms p22phox, p67phox, and gp91phox. It also increased glutathione levels and SOD activity and reduced nitrotyrosine production. These protective effects of CBD against oxidative/nitrative stress were also demonstrated *in vitro* in human primary cardiomyocytes[95].

In a study published in 2017, Vella *et al*[96] found that giving cannabinoids to diabetic rats reversed changes in lipid peroxidation and oxidative stress markers, as well as blocking maladaptive alterations in the structure and function of the heart and blood vessels. Similar findings were previously published by Rajesh's group, who reported that administering CBD to diabetic C57BL/6J mice for 11 wk reduced the formation of lipid peroxides, protein carbonyls, and ROS in the heart[95]. Furthermore, the binding site of anandamide has been linked to NO release[97], implying a possible mechanism by which cannabinoids could increase NO bioavailability. THC treatment of STZ-induced diabetic rats resulted in a controlled redox state that granted improvements in end organ function of the myocardium and vasculature[96]. This was demonstrated by preservation of myocardial pump function, cardiac electrophysiology, noradrenergic-mediated contraction, and endothelial-dependent relaxation of resistance arteries. These findings suggested that cannabinoid receptor activation in an experimental type I diabetes animal might be a potential pharmacological target for diabetic cardiomyopathy management [96] (Table 2).

Inflammation

Inflammation is a complex nonspecific response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants, and it involves several functional and molecular mediators, such as the recruitment and activation of leukocytes such as mast cells, neutrophils, and monocytes/macrophages. On an acute basis, inflammation is usually good since it represents the organism's defensive attempt to eliminate damaging stimuli and begin the healing process. Inflammation, on the other hand, might have negative consequences if it continues for a long period[98]. The increased expression of many inflammatory proteins is regulated at the level of gene transcription through the activation of pro-inflammatory transcription factors which play a critical role in amplifying and perpetuating the inflammatory process[99].

Activation of the transcription factor nuclear factor-kappa B (NF-κB), which binds to DNA and activates gene transcription, appears to play a pivotal role in the regulation of inducible enzymes such as inducible nitric oxide synthase (iNOS), inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, prostaglandins, cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), in addition to other substances that are initiators or enhancers of the inflammatory process[100,101]. The aforementioned inflammatory mediators bind to specific target receptors on the cells and may increase vascular permeability, promote inflammatory cell chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain, and/or mediate oxidative damage[102].

Numerous investigations have indicated that inflammatory processes play a critical role in the development of diabetes macro- and microvascular complications[29,103]. Cardiac inflammation is a common and early symptom of diabetes, and it plays a key role in the progression of heart failure in diabetic cardiomyopathy[104]. Furthermore, various research on the heart of diabetic or diabetic hypertensive rats has shown that NF-κB plays a major role in the development of diabetic cardiomyopathy[105,106].

Cannabinoid receptor expression in immune cells can be influenced by various inflammatory factors and other triggers activating these cells[107]. Inflammatory stimuli may potentially boost the synthesis of endocannabinoids in immune cells (*e.g.*, macrophages, monocytes, and dendritic cells) by activating

Table 2 Summary of possible mechanisms by which cannabinoids and the endocannabinoid system could modulate diabetic cardiomyopathy

Cannabinoid agent	Mechanism	Effect
Endocannabinoids	Oxidative/Nitrative stress	Influenced ROS and RNS production[28]
	Myocardial remodeling	Triggered activation of signaling pathways (*e.g.*, p38 and JNK-MAPKs), promoting cell death[50,137]
	Inflammation	Increased during inflammation[107]
		Modulating T and B lymphocyte proliferation and apoptosis, inflammatory cytokine production and immune cell activation by inflammatory stimuli[107,108,111]
AM281	Oxidative/Nitrative stress	Attenuated doxorubicin-induced oxidative stress[52]
SR141716A	Oxidative/Nitrative stress	Attenuated doxorubicin-induced oxidative stress[52]
	Inflammation	Reduced plasma levels of the pro-inflammatory cytokines MCP-1 and IL-12 in low density lipoprotein deficient mice[113]
		Inhibited LPS-induced pro-inflammatory IL-6 and TNF-α expression[113]
	Myocardial remodeling	Reduced activation of p38 and JNK/MAPK[90]
		Improved myocardial dysfunction induced in a mouse model of diabetic cardiomyopathy[92]
		Reduced markers of cell death (activated caspase-3 and chromatin fragmentation)[92]
JWH133	Oxidative/Nitrative stress	Reduced ROS release in ApoE knockout mice[54]
	Inflammation	Decreased leukocyte recruitment in ApoE-knockout mice[54]
		Attenuated TNF-α-induced NF-κB activation[116]
		Attenuated ICAM-1 and VCAM-1 up-regulation[116]
Cannabidiol	Oxidative/Nitrative stress	Attenuated oxidative and nitrative stress in the myocardium of streptozotocin-induced diabetic mice[93]
		Prevented changes in markers of lipid peroxidation and oxidative stress in diabetic rats[96]
	Inflammation	Inhibited IκB-α phosphorylation and subsequent p65 NF-κB nuclear translocation[93]
		Attenuated high glucose-induced NF-κB activation in primary human cardiomyocytes[93]
	Myocardial remodeling	Attenuated the established systolic and diastolic dysfunction in diabetic mice[93]
		Attenuated the activation of stress signaling pathways: p38 and JNK/MAPKs[93]
		Enhanced the activity of the pro-survival AKT pathway in diabetic myocardium[93]
		Decreased the activity of the pro-apoptotic enzyme caspase-3[93]
	Autophagy	Promoted endothelial cell survival *via* HO-1 mediated autophagy[170]
Anandamide	Oxidative/Nitrative stress	Induced NO bioavailability[97]
	Myocardial remodeling	Decrease rat heart mitochondrial O_2 consumption[135]
		Increased activation of p38 and JNK/MAPK, followed by cell death[90]
		Enhanced doxorubicin-induced MAPK activation and cell death[90]
Δ 9-tetrahydrocannabinol (THC)	Oxidative/Nitrative stress	Regulated redox state in diabetic rats[96]
	Myocardial remodeling	Decreased rat heart mitochondrial O_2 consumption[135]
WIN55, 212-2	Inflammation	Reduced atherosclerotic lesion macrophage content and IL-6 and TNF-α levels[114,115]
		Reduced adhesion molecules VCAM-1 and ICAM-1 as well as NF-κB activation[114,115]

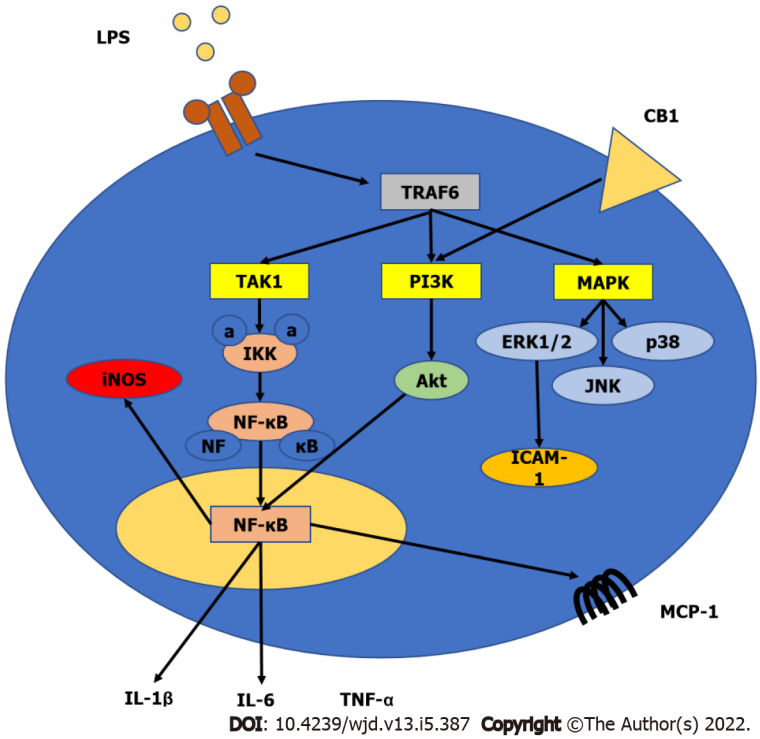
HU-308	Inflammation	Attenuated TNF-α-induced NF-κB activation, ICAM-1 and VCAM-1 up-regulation[116]
		Decreased endothelial cell activation and suppression of the acute inflammatory response[56,117]
	Autophagy	Enhanced autophagy levels in heart tissues with diabetic cardiomyopathy [171]
		Increased AMPK phosphorylation while decreasing the phosphorylation of mTOR[171]
HU-210	Myocardial remodeling	Decrease rat heart mitochondrial O_2 consumption[135]
		Increased activation of p38 and JNK/MAPK, followed by cell death[90]
		Enhanced doxorubicin-induced MAPK activation and cell death[90]
		Enhanced left ventricular performance in rats with myocardial infarction [143]
AM251	Myocardial remodeling	Improved cardiac function in carbon tetrachloride-induced cirrhosis in rats[140]
		Reduced activation of p38 and JNK/MAPK[90]

LPS: Lipopolysaccharide; AMPK: Adenosine monophosphate activated protein kinase; mTOR: Mammalian target of rapamycin; IL: Interleukin; JNK: Jun N-terminal kinase; MAPK: Mitogen activated protein kinases; TNF-α: Tumor necrosis factor-α; NF-κB: Nuclear factor-kappa B; ICAM-1: Intercellular adhesion molecule-1; VCAM-1: Vascular cell adhesion molecule-1.

multiple biosynthetic pathways and/or decreasing the expression of metabolic enzymes that degrade them[107,108]. THC and other natural or synthetic cannabinoids have been studied for their immunomodulatory effects in mice and/or rats *in vivo*, as well as in cultured human immune cells. Overall, cannabinoid ligands exhibit suppressive effects on B-lymphocytes, T-lymphocytes, natural killer cells, and macrophages[109,110], which are most likely due to both CB1 and CB2 receptor-dependent and –independent mechanisms. Other studies have revealed that endocannabinoids can influence immune functions by modulating T and B lymphocyte proliferation and apoptosis, inflammatory cytokine production and immune cells activation in response to inflammatory stimuli, macrophage-mediated killing of sensitized cells, chemotaxis, and inflammatory cell migration[107,110,111]. Furthermore, cannabinoids may influence the expression of iNOS and the formation of ROS in immune cells, which play significant roles in the defense against invading pathogens and in modulation of the inflammatory response[21]. The involvement of cannabinoid receptors in inflammation is shown in Figure 3.

Han *et al*[112] demonstrated that CB1 receptors promote pro-inflammatory responses of macrophages through ROS production, and subsequent synthesis of TNF-α and monocyte chemoattractant protein-1 (MCP-1). This effect was negatively regulated by CB2 and was attenuated by CB1 blockade. In a mouse model of atherosclerosis, the CB1 antagonist SR141716A (rimonabant) was able to reduce plasma levels of the pro-inflammatory cytokines MCP-1 and IL-12 in low density lipoprotein deficient mice fed with a high fat diet[113]. In addition, rimonabant inhibited lipopolysaccharide (LPS)-induced pro-inflammatory IL-6 and TNF-α expression in mouse peritoneal macrophages *in vitro*. Importantly, this effect was still observed when cells from CB1-knockout mice were used, suggesting a CB1-independent anti-inflammatory effect of rimonabant[113]. In another model of atherosclerosis, Hoyer and co-workers demonstrated a severe vascular leukocyte infiltration in ApoE and CB2 double knockout mice which was more intense than that observed in ApoE-knockout mice[54]. Interestingly, treatment with the selective CB2 agonist JWH-133 decreased leukocyte recruitment in ApoE-knockout mice compared to their wild-type counterparts[54]. In 2010, Zhao *et al*[114,115] showed that treatment with the synthetic cannabinoid WIN55,212-2 reduced atherosclerotic lesion macrophage content and mRNA levels of inflammatory markers IL-6 and TNF-α; adhesion molecules VCAM-1 and ICAM-1 as well as NF-κB activation in ApoE-deficient mice fed on high-cholesterol diets. In human coronary artery endothelial cells, activation of CB2 receptors with the selective agonists HU-308 or JWH-133 attenuated the TNF-α-induced NF-κB activation, ICAM-1 and VCAM-1 up-regulation, MCP-1 release, as well as trans-endothelial migration and adhesion of monocytes, which are hallmarks of the development of atherosclerosis[116].

The beneficial effects of CB2 receptor activation by selective synthetic ligands, such as JWH-133 and HU-308, was largely attributed to decreased endothelial cell activation and suppression of the acute inflammatory response in animal models of myocardial ischemia/reperfusion injury, which are characterized by a rapid increase in cytokines and chemokines in addition to an enhanced influx of leukocytes into the vulnerable region. Attenuated expression of adhesion molecules, chemokine secretion, leukocyte chemotaxis, adherence to endothelium, stimulation of trans-endothelial migration, and linked oxidative/nitrative stress associated with reperfusion damage were all beneficial effects of CB2 receptor



DOI: 10.4239/wjd.v13.i5.387

Figure 3 Role of cannabinoid receptors in inflammation. Activation of toll-like receptor 4 induces tumor necrosis factor receptor associated factor 6, which activates transforming growth factor beta-activated kinase 1 (TAK1), phosphoinositide 3-kinase (PI3K), and mitogen-activated protein kinase signaling. This will induce the formation of nuclear factor-kappa B (NF-κB) and the subsequent increase in inflammatory cytokines levels such as interleukin 6 (IL-6), IL-1β, and tumor necrosis factor α (TNF-α) in addition to the activation of inducible nitric oxide synthase (iNOS) and monocyte chemoattractant ptotein-1 (MCP-1). Stimulation of cannabinoid receptors will increase the PI3K activity leading to increased activity of protein kinase B (Akt/PKB), which in turn enhances the nuclear translocation of NF-κB. TLR4: Toll-like receptor 4; TRAF6: Tumor necrosis factor receptor associated factor 6; PI3K: Phosphoinositide 3-kinase; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor-kappa B; IL: Interleukin; TNF-α: Tumor necrosis factor α; MCP-1: Monocyte chemoattractant protein-1.

activation[56,117].

In a mouse model of streptozotocin-induced diabetic cardiomyopathy, which is characterized by up-regulation of the expression of various inflammatory cytokines in the myocardium, genetic deletion, or pharmacological blockade of CB1 receptors resulted in attenuation of the expression of: Inflammatory cytokines such as TNF-α and IL-1β, adhesion molecules such as ICAM-1 and VCAM-1, iNOS, and cyclooxygenase 2 (COX2)[92]. In another study using the same model of diabetic cardiomyopathy, it was shown that there was a marked phosphorylation of the inhibitor of NF-κB (IκB-α) in the cytosol of diabetic hearts, leading to the release of the active p65 subunit of NF-κB, which subsequently translocated to the nucleus to induce the expression of inflammatory and apoptotic genes[95]. Treatment with CBD, the non-psychoactive cannabinoid, inhibited the IκB-α phosphorylation and subsequent p65 NF-κB nuclear translocation. The CBD treatment also inhibited the NF-κB-dependent mRNA and/or protein expression of adhesion molecules (ICAM-1 and VCAM-1), the pro-inflammatory cytokine TNF-α, and iNOS in the diabetic myocardial tissues. Cannabidiol (CBD) was also able to attenuate high glucose-induced NF-κB activation in primary human cardiomyocytes[95] (Table 2).

Accumulation of AGEs

An important consequence of high glucose-induced cellular injury is the formation of AGEs. AGEs are a heterogeneous group of compounds formed by the non-enzymatic glycation reaction of glucose and other glycating compounds with proteins and, to a lesser extent, lipids, and DNA[118]. In addition, AGEs can easily make covalent cross-linkages (adducts) with macromolecules like proteins and, in this way, can change the structure and function of these proteins[119]. In diabetic patients, the rate of formation of AGEs is increased. Thus, over time, even modest hyperglycemic excursions can result in significant adduct accumulation in long-lived macromolecules[120]. It is now well established that AGEs interact with cell surface receptors and binding proteins to evoke varied downstream responses. These include the pro-inflammatory responses that could play a critical role in the pathogenesis of diabetic complications including cardiomyopathy[121]. The receptor for AGEs (RAGE) is the most established and the best characterized AGE binding protein[122]. The RAGE is a trans-membrane receptor that belongs to the immunoglobulin super-family and is constitutively expressed in a range of tissues including neurons, endothelium, smooth muscle, epithelium, and inflammatory cells[123].

There are two basic methods by which AGEs might alter myocardial function. AGEs, for starters, can form covalent adducts with proteins including collagen, laminin, and elastin[118,124]. As shown in the myocardium of an animal model of type 2 diabetes, this can inhibit collagen degradation, resulting in collagen buildup and fibrosis, producing increased myocardial stiffness, and reduced ventricular relaxation[19]. Second, soluble extracellular AGEs can bind to RAGE, causing up-regulation of transforming growth factor–β (TGF–β) and NADPH oxidase, resulting in the generation of substantial quantities of cytoplasmic and extracellular superoxide, which can then interact with NO to produce RNS[118]. Furthermore, when the RAGE receptor is active, it promotes the transcription factor NF-κB and associated genes by elevating intracellular free radical levels and by triggering multiple other signaling pathways[125].

In the literature, little is known about the interaction of the endocannabinoid system and the AGE and/or RAGE. In a mouse model of streptozotocin-induced diabetic cardiomyopathy, genetic deletion of CB1 receptors attenuated accumulation of AGEs and the expression of RAGE in the myocardium of diabetic mice[92] (Table 2).

Myocardial remodeling

Diabetes has a multifactorial nature, therefore there are changes at the cellular and molecular levels that predispose the heart to pathological, structural, and functional remodeling[4]. Diabetic cardiomyopathy is characterized by an unusually increased left ventricular mass and myocardial fibrosis. Left ventricular hypertrophy has been associated with hyperinsulinemia, insulin resistance, increased non-esterified fatty acids, and activation of the renin-angiotensin-aldosterone system[82]. Chronic cardiac remodeling and structural alterations are promoted by a continual cycle of increased ROS production[126,127]. Diastolic dysfunction is defined as an elevation in ventricular wall stiffness and prolonged diastolic relaxation time, and it is common in the early stages of cardiomyopathy[128].

Increased triglyceride buildup and decreased calcium absorption have been linked to diastolic dysfunction[128]. The progression of systolic dysfunction is marked by dilated cardiac remodeling, which leads to heart failure[129]. Cardiomyocyte mortality is accompanied by fibroblast replacement, which leads to interstitial fibrosis driven predominantly by TGF-β[130]. Cardiomyocyte death is mediated by activation of various stress signaling pathways and consequent apoptosis. The deleterious effect of accumulating free fatty acids on mitochondrial biogenesis eventually leads to mitochondrial apoptosis and lowered ATP generation, which is insufficient to meet cardiac demands, resulting in impaired cardiac contractility and lowered ejection fraction[128]. Myocardial dysfunction is caused by impaired endothelial function linked with insulin resistance[13].

Cannabinoid receptors are believed to have the ability to control apoptosis since they may signal through both pro- and anti-apoptotic pathways. Due to the lipophilic nature of their structures, they may be able to operate intracellularly without the help of a membrane transporter[131]. A number of cannabinoid drugs, including HU-210, THC, and anandamide, have been demonstrated to reduce cardiac mitochondrial O2 consumption in rats[132], as well as the role of mitochondria in marijuana-induced cell death[133]. Stimulation of CB1 receptors by endocannabinoids has also been linked to the activation of signaling pathways (*e.g.*, p38 and JNK-MAPKs) and cell death in various clinical circumstances[51,134]. It is reasonable to conclude, based on earlier results and observations of reduced cardiac apoptosis in FAAH-null animals, that endocannabinoids have strong potential for the regulation of apoptosis, and hence remodeling, in the heart[135].

Doxorubicin treatment is linked to increased anandamide levels in the myocardium, but not to alterations in CB1 or CB2 receptor expression[52]. Doxorubicin triggered apoptosis in a cardiac cell line (H9c2) that was reduced by CB1 receptor blockage, but the result was not sensitive to a CB2 blocker or CB1 and CB2 receptor agonists[52]. Similarly, studies on cardiac function suggest that endocannabinoids have a role in cirrhosis-related cardiac dysfunction[136]. AM251, which blocks CB1 receptors, enhanced cardiac function in rats with carbon tetrachloride-induced cirrhosis, and anandamide levels were shown to be elevated in the hearts of cirrhotic rats compared to controls[137]. In contrast, aging-associated cardiac dysfunction is reduced in FAAH-null mice, which could be interpreted as showing a need for increased endocannabinoid activity in the heart[135].

Mukhopadhyay *et al*[90] demonstrated that genetic deletion of CB1 receptors attenuated cardiac dysfunction induced by doxorubicin in mice. In this study, doxorubicin-induced activation of stress signaling pathways (p-38 and JNK/MAPKs) with subsequent apoptosis was attenuated in CB1 knockout mice. In addition, these findings were supported *in vitro* in human primary cardiomyocytes as the activation of CB1 receptors by anandamide or HU210 resulted in increased activation of p38 and JNK/MAPK, followed by cell death, which are effects that were attenuated by both selective CB1 antagonists (SR141716A or AM281) and MAPK inhibitors[90]. Furthermore, doxorubicin-induced MAPK activation and cell death in human cardiomyocytes were significantly enhanced when doxorubicin was co-administered with anandamide or HU210, an effect which could also be attenuated by both CB1 antagonists and MAPK inhibitors[90]. Another aspect of doxorubicin-induced cardiotoxicity is the induction of myocardial fibrosis, an effect that was attenuated by genetic deletion of CB1 indicating its role in this model of cardiotoxicity[90]. In another study using the same model of doxorubicin-induced cardiotoxicity in mice, it has been shown that FAAH knockout mice exhibited significantly increased doxorubicin-induced cardiac dysfunction and myocardial cell death compared to

their wild-type counterparts. The effects of doxorubicin in FAAH knockouts were attenuated by CB1 receptor antagonists[91].

Acute myocardial infarction causes cardiomyocyte necrosis, which triggers repair mechanisms that result in scarring[138]. This post-infarction cardiac remodeling process involves adaptive changes in the ventricular shape, size, and function, which can lead to contractile dysfunction and heart failure[138]. In ischemic cardiomyocyte death, fibrosis, and cardiac dysfunction, Defer and coworkers showed considerable evidence for the protective impact of CB2 receptors[139]. CB2-knockout mouse hearts displayed larger infarcts and more persistent cell loss 3 d after ischemia, as well as accelerated damage and apoptosis in the non-ischemic remote myocardium compared to wild-type mice[139]. Cardiomyocytes and fibroblasts lacking CB2 were more vulnerable to oxidative stress-induced cell death *in vitro*. Long-term effects of cardiac remodeling in CB2-knockout hearts involved marked fibrosis, accelerated cardiomyocyte hypertrophy, dilated cardiomyopathy, and cardiac dysfunction, as reported 4 wk post-infarction[139]. On other hand, wild-type post-ischemic hearts acquired mild fibrosis and cardiomyocyte hypertrophy while maintaining cardiac function[139]. Wagner and colleagues revealed another investigation where the administration of the CB1 antagonist AM251 for 12 wk after an experimentally induced infarction exacerbated the decline in left ventricular function, but administration of the non-selective cannabinoid agonist HU-210 improved left ventricular performance[140].

A previous study conducted by Liao and co-workers demonstrated that CB1 deficiency contributed to the exacerbation of chronic cardiac remodeling induced by pressure overload in mice, revealing a new role of CB1 in the pathophysiology of congestive heart failure[141]. Genetic deletion of CB1 was found to worsen left ventricular hemodynamics and exacerbate cardiac hypertrophy compared to wild-type mice. Furthermore, it was found that CB1 deficiency led to enhanced activation of the epidermal growth factor receptor, p38, and ERK/MAPKs, which contributed to the exacerbation of cardiac hypertrophy [141].

In patients with chronic heart failure, clinical data revealed an increase in cardiac CB2 expression as well as increased levels of the endocannabinoids, anandamide, and 2-AG[142]. Additionally, in these patients, cannabinoid receptor expression was also found to be slightly downregulated[142]. It was believed that CB2 up-regulation could have a negative inotropic effect due to lower cyclic adenosine monophosphate (cAMP) levels, which could lead to ventricular weakness. CB2 receptors, on the other hand, may mediate positive inotropic effects *via* cAMP-independent processes, hence serving as a compensation strategy to sustain heart function[53]. Furthermore, as demonstrated in rats, CB2 upregulation could be a protective response to counteract structural alterations caused by chronic heart failure [139]. Recently, it has been shown that in biopsies collected from the hypertrophic myocardium of patients with aortic stenosis, there were elevated concentrations of anandamide, higher expression of its degrading enzyme FAAH, and of CB2 receptors[143].

Rajesh *et al*[92] indicated that myocardial dysfunction induced in a mouse model of diabetic cardiomyopathy was improved in CB1-knockout mice or in diabetic mice treated with CB1 antagonists (SR141716A or AM281). This was demonstrated by improved indices of left ventricular systolic and diastolic dysfunction, ejection fraction, contractility, and ventricular stiffness. In the same study, there was attenuated activity of MAPKs and reduced markers of cell death (activated caspase-3 and chromatin fragmentation) in the myocardium of diabetic CB1-knockout mice and in diabetic wild-type mice treated with the CB1 antagonist (SR141716A). Diabetic mice developed myocardial fibrosis as a structural consequence of diabetic cardiomyopathy, and this was characterized by increased accumulation of collagen and enhanced expression of markers of fibrosis such as TGF-β and fibronectin. Interestingly, these changes were attenuated by genetic deletion or pharmacological blockade of CB1 receptors[92]. In another study using the same model, chronic treatment of diabetic mice with the non-psychoactive CBD attenuated the established systolic and diastolic dysfunction in diabetic mice[95]. In addition, CBD treatment attenuated the activation of stress signaling pathways: p38 and JNK/MAPKs. It also enhanced the activity of the pro-survival AKT pathway in diabetic myocardium. Another beneficial effect of CBD treatment in this model was its ability to decrease the activity of the pro-apoptotic enzyme caspase-3 and to reduce the rate of cell death in diabetic myocardium. Finally, CBD treatment protected diabetic myocardium from the deleterious process of fibrosis by decreasing myocardial collagen content and attenuating the expression of fibrosis markers: TGF-β, fibronectin, and the enzyme matrix metalloproteinase[95] (Table 2).

Autophagy

Autophagy, an essential metabolic process, is a self-degradative and recycling procedure dependent on lysosomes. It targets dysfunctional organelles and long-lived proteins[144,145]. This occurs through the biogenesis of double-membrane vesicles containing cytoplasmic components destined for lysosomal degradation, these vesicles are known as autophagosomes[146]. Autophagosome biogenesis entails nucleation, expansion, and closure of the phagophore (a cup-shaped membrane) thereby sequestering cytoplasmic cargo. This is followed by fusion with endolysosomal compartments to facilitate degradation of the sequestered material[146].

Autophagy is performed by genes called autophagy-related (ATG) genes[147]. The discovery of ATG genes in yeast in the 1990s allowed researchers to identify how autophagy works[148]. Today, 36 ATG proteins have been identified as being particularly significant for autophagy, with 18 of them belonging

to the basic machinery[149]. Through the Unc-51-like kinases, ULK1 and ULK2 (mammalian homologues of ATG1), two protein kinases (mTOR and AMPK) control autophagy in mammals[150]. The ULK kinases are dephosphorylated and activated when autophagy is induced. Beclin-1 (mammalian ortholog of ATG6), which is part of a protein complex, is phosphorylated and activated by the ULK[151]. The active ULK and Beclin-1 complexes translocate to the phagophore, the site of autophagosome initiation, where they both help to stimulate downstream autophagy components[152].

Autophagosome production requires two ubiquitin-like conjugation mechanisms[153]. The first one covalently binds the ubiquitin-like protein ATG12 to ATG5. The conjugate protein subsequently attaches to ATG16L1, forming an E3-like complex that is part of the second ubiquitin-like conjugation system[154]. This complex binds and activates ATG3, which covalently binds to the mammalian homologues of the ubiquitin-like yeast protein LC3 to the lipid phosphatidylethanolamine (PE) on autophagosome surfaces[155]. Lipidated LC3 aids autophagosome closure[156] and facilitates the docking of particular cargos and adaptor proteins such as Sequestosome-1/p62[157]. The autophagosome then unites with a lysosome to produce an autolysosome. The autolysosome's contents are then destroyed, and their constituents are liberated from the vesicle[158].

Autophagy has a role in the control of cardiovascular disorders such as myocardial infarction and atherosclerosis[159]. Increased autophagy levels have been shown to protect against diabetic cardiomyopathy[160,161]. As a result, pharmacological activation of autophagy might be a promising therapeutic strategy for diabetic cardiomyopathy.

Autophagy also plays an essential role in the functioning of a variety of receptors. In the case of cannabinoid receptors, autophagy has been related to the protective effects of CB2 in a variety of disorders[162-164], suggesting the relevance of autophagy in disease treatment. Autophagy was previously shown to contribute to the alleviative effects mediated by CB2 activation in inflammatory disorders such as multiple sclerosis, alcoholic liver disease, and inflammatory bowel disease[162-164]. Activating CB2 improved inflammatory bowel disease in mouse models by inhibiting the NLRP3 inflammasome and triggering autophagy in murine macrophages, according to Ke and colleagues[163]. In mouse multiple sclerosis models, a similar relationship between CB2 and autophagy was discovered [164].

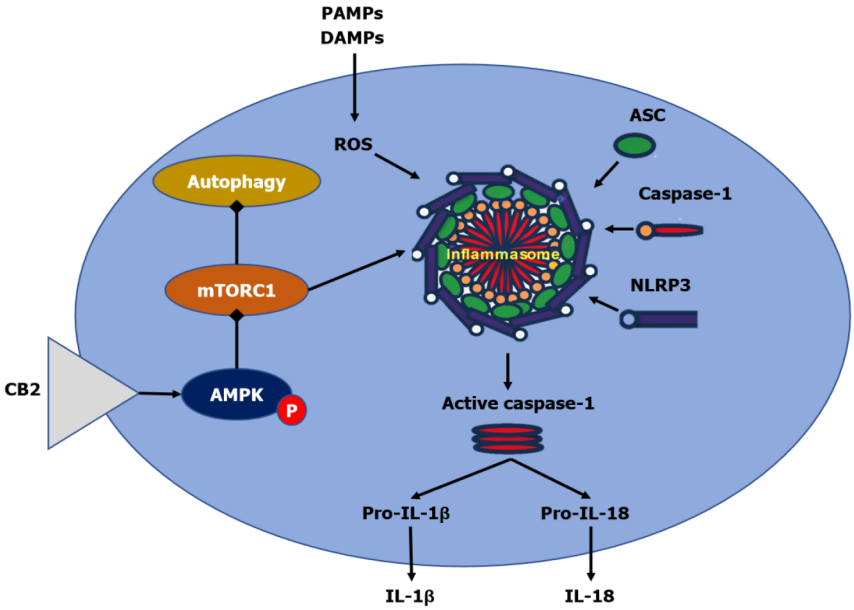
In the case of autophagy in diabetic cardiomyopathy, it was shown that increasing autophagy levels contributed to improving the condition. Several treatments have been demonstrated to be beneficial in reducing the etiology and development of diabetic cardiac myopathy by utilizing enhanced autophagy levels[160,161,165,166]. CB2 activation *via* autophagy induction provided protection against diabetic cardiomyopathy, according to a recent study by Wu and coworkers[167]. They used HU308 to selectively activate CB2, resulting in a substantial increase in autophagy levels in diabetic cardiomyopathy heart tissues *in vivo* and hyperglycemia-challenged cardiomyocytes *in vitro*. Furthermore, inhibiting autophagy with bafilomycin A1 reduced the cardioprotective effect of HU308 in both *in vitro* and *in vivo* models. Wu *et al*[167] concluded that CB2-induced autophagy was involved in the CB2-mediated cardio-protective effect.

Resveratrol, an autophagy inducer, was discovered to have a cardio-protective impact in cardiomyocytes exposed to hyperglycemia *via* the AMPK-mTOR-p70S6K signaling pathway[168]. AMPK-mTOR signaling contributed to the cardio-protective effect in STZ-induced diabetic mice by increasing autophagy[161,169]. According to these findings, Wu *et al*[167] found that administering HU308 to selectively activate CB2 enhanced AMPK phosphorylation while lowering mTOR and p70S6K phosphorylation, initiating the AMPK-mTOR-p70S6K signaling cascade in murine primary ventricular cardiomyocytes. Furthermore, using compound C, an AMPK inhibitor, significantly reduced the cardioprotective effect of HU308, showing that AMPK-mTOR-p70S6K signaling-induced autophagy was essential in CB2-mediated cardiac protection in dilated cardiomyopathy[167]. However, because the mechanisms behind CB2-mediated autophagy activation are complex, more research is required. Figure 4 summarizes the effect of cannabinoid receptors on AMPK/mTORC1/NLRP3 signaling.

Cannabidiol (CBD), has recently gained increased interest for therapeutic use. Indeed, CBD has been shown to suppress a high glucose-induced inflammatory response and barrier disruption of endothelial cells[170] and to attenuate myocardial dysfunction, cardiac fibrosis, oxidative/nitrative stress, inflammation, cell death, and interrelated signaling pathways in a mouse model of type I diabetic cardiomyopathy[95]. The critical role of HO-1 has been evident in the regulation of autophagy, with survival-enhancing effects in various cell types, including endothelial cells[171-173]. Moreover, HO-1 showed positive *in vivo* effects in animal models of atherosclerosis and restenosis[174]. Böckmann and Hinz have recently proved that CBD promoted endothelial cell survival *via* HO-1 mediated autophagy[170] (Table 2).

CONCLUSION

Diabetes-induced cardiomyopathy is a deleterious complication of the cardiovascular system characterized by structural and functional changes in the myocardium that ultimately lead to cardiac failure. The mechanisms underlying the development of diabetic cardiomyopathy are complex and involve several pathogenic pathways. A great body of evidence supported a special role of oxidative/nitrative



DOI: 10.4239/wjd.v13.i5.387

Figure 4 Effect of cannabinoid receptors on adenosine monophosphate activated protein kinase/mammalian target of rapamycin complex 1/NLR family pyrin domain containing 3 signaling. Cannabinoids enhance the phosphorylation of adenosine monophosphate activated protein kinase (AMPK), which reduces the stimulatory effect of mammalian target of rapamycin complex 1 (mTORC1) on inflammasome assembly. Depressed activation of NLR family pyrin domain containing 3 (NLRP3) will diminish the activation of procaspase-1 leading to a decrease in interleukin-1β (IL-1β) and IL-18 production. Additionally, the inhibitory effect of phosphorylated AMPK on mTORC1 will enhance autophagy. AMPK: Adenosine monophosphate activated protein kinase; mTORC1: Mammalian target of rapamycin complex 1; NLRP3: NLR family pyrin domain containing 3; IL: Interleukin.

stress and inflammation in the pathogenesis of diabetic cardiomyopathy. The endocannabinoid system has been implicated in the development of several pathological conditions including cardiovascular disorders. Several mechanisms have been proposed as targets by which cannabinoids and the endocannabinoid system could modulate cardiovascular disorders and recent evidence suggested the involvement of this system in the pathogenesis of diabetic cardiomyopathy. Indeed, the manipulation of the endocannabinoid system could represent a promising therapeutic approach for diabetic cardiomyopathy, and several mechanisms have been proposed for this role including its effects on oxidative/nitrative stress, inflammatory pathways, and autophagy together with possible effects on cardiac remodeling. However, more research is needed to define the exact mechanisms of the intervention of the different components of this system in diabetic cardiomyopathy.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Moshira Ibrahim; Professor of Pharmacology at Al-Azhar University (Girls), for her sincere guidance and supervision of AEW during writing the initial draft.

FOOTNOTES

Author contributions: El-Azab MF was responsible for conceptualization and supervision; El-Azab MF and Wakiel AE were responsible for original draft preparation; El-Azab MF, Nafea YK and Youssef ME were responsible for review of the literature and visualization; all authors contributed to the final draft of the manuscript, have read and agreed to the published version of the manuscript.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Country/Territory of origin: Egypt

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S-Editor: Fan JR
L-Editor: Webster JR
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 May 15; 13(5): 408-416

DOI: 10.4239/wjd.v13.i5.408

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Changes and significance of retinal blood oxygen saturation and oxidative stress indexes in patients with diabetic retinopathy

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Lemmen KD, Germany; Ortega AL, Spain; Sasongko MB, Indonesia

Received: December 13, 2021
Peer-review started: December 13, 2021
First decision: January 12, 2022
Revised: January 28, 2022
Accepted: April 28, 2022
Article in press: April 28, 2022
Published online: May 15, 2022

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Abstract

BACKGROUND

Diabetic retinopathy (DR) is a diabetic complication that can severely affect the patients' vision, eventually leading to blindness. DR is the most important manifestation of diabetic micro-vasculopathy and is mainly related to the course of diabetes and the degree of blood glucose control, while the age of diabetes onset, sex, and type of diabetes have little influence on it.

AIM

To explore the changes in blood oxygen saturation and oxidative stress indices of retinal vessels in patients with DR.

METHODS

In total, 94 patients (94 eyes) with DR (DR group) diagnosed at Jianyang people's Hospital between March 2019 and June 2020, and 100 volunteers (100 eyes) (control group) without eye diseases, were included in this study. Arterial and venous blood oxygen saturation, retinal arteriovenous vessel diameter, and serum oxidative stress indicators in the two groups were compared. Based on the stage of the disease, the DR group was divided into the simple DR and proliferative DR groups for stratified analysis.

RESULTS

The oxygen saturation of the retinal vessels in the DR group was significantly higher than that in the control group ($P < 0.05$). The retinal vessel diameters between the DR and control groups were not significantly different. The serum malondialdehyde (MDA) and 8-hydroxydehydroguanosine (8-OHdG) levels in the DR group were significantly higher than those in the control group ($P < 0.05$). The serum superoxide dismutase (SOD) and reduced glutathione (GSH) levels in

the DR group were significantly lower than those in the control group ($P < 0.05$). The oxygen saturation of the retinal vessels in the patients with proliferative DR was significantly higher than that in the patients with simple DR ($P < 0.05$). The retinal vessel diameter in patients with proliferative DR was not significantly different from that of patients with simple DR ($P > 0.05$). Serum MDA and 8-OHdG levels in patients with proliferative DR were significantly higher than those in patients with simple DR ($P < 0.05$). Serum SOD and GSH levels in patients with proliferative DR were significantly lower than those in patients with simple DR ($P < 0.05$).

CONCLUSION

Increased blood oxygen saturation of retinal arteries and veins and increased oxidative stress damage in patients with DR may be associated with decreased retinal capillary permeability and arterial oxygen dispersion, possibly reflecting the patient's condition.

Key Words: Diabetes; Retinopathy; Diabetic retinopathy; Blood oxygen saturation; Oxidative stress

Core Tip: Diabetic retinopathy (DR) is a complication of diabetes. Studies have shown that retinal blood oxygen saturation and oxidative stress are closely associated with hypoxic-ischemic injury of retinal tissues caused by DR. Although DR treatment has improved in recent years, the long-term prognosis for late DR is not optimistic, and effective methods are needed to prevent DR from deteriorating in the later stages. Therefore, determining the incidence of DR and establishing its early diagnosis are considered clinically important. Our study monitored and analyzed retinal blood vessels, reflecting changes in serum biological indicators of blood oxygen saturation and oxidative stress levels in patients with DR, to determine the patient's condition, thus improving the existing diagnosis and treatment methods and developing new methods for the treatment of serious complications of diabetes and subsequently ameliorating the cure rate of patients.

Citation: Wang XL, Cai FR, Gao YX, Zhang J, Zhang M. Changes and significance of retinal blood oxygen saturation and oxidative stress indexes in patients with diabetic retinopathy. *World J Diabetes* 2022; 13(5): 408-416

URL: https://www.wjgnet.com/1948-9358/full/v13/i5/408.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i5.408

INTRODUCTION

Diabetic retinopathy (DR) is one of the main complications of diabetes and includes ophthalmic and microvascular lesions caused by abnormal insulin metabolism in patients with diabetes. This can lead to impaired ocular nutrition and visual function, which, in turn, can lead to adult blindness[1,2], making DR one of the main causes of vision impairment and blindness globally. The incidence of retinal complications can increase with disease duration. Over time, up to 50% of people with type 1 diabetes and 30% of people with type 2 diabetes may potentially develop visually threatening retinal changes, with symptoms that are not evident during the early stage[3,4]. High blood glucose level, hypertension, renal disease, and hyperlipidemia are all typical diseases associated with diabetes, which are related to the pathogenesis of DR. Moreover, in diabetes, the pathological cascade of blood vessel injury can be caused by high blood glucose level and the related oxidative stress[5]. Due to the subsequent vascular wall interference, the permeability of the blood-retinal barrier is disrupted, which leads to hypoxia and eventually, retinal nutrition degeneration and photoreceptor cell death. The subsequent progression of retinopathy leads to retinal neovascularization, vitreous hemorrhage, and fibrous tissue formation in the preretinal hemorrhage focus[6]. Although the treatment for DR has improved in recent years, the long-term prognosis for late DR remains poor, and it is necessary to determine effective ways to prevent progression of DR in the later stages. Further, despite the transparency regarding the pathogenesis and treatment of DR in recent years, several questions remain unanswered. Currently, most DR treatments are mainly focused on the later stage, failing to address the early, potentially reversible stage of the disease; moreover, patients in the late stage have more complications. Therefore, determining the incidence of DR and establishing its early diagnosis have important prognostic and scientific values, which may help to improve the existing methods and provide new methods for the diagnosis and treatment of diabetes with severe complications.

According to recent studies[7-9], hypoxic-ischemic injury of retinal tissues caused by diabetic microangiopathy is closely associated with retinal blood oxygen saturation. Retinal blood oxygen saturation is associated with disease severity and is significantly higher in patients with DR than in normal individuals. The human retina, an energy-demanding organ that is particularly sensitive to reactive oxygen species and lipid peroxides, is rich in polyunsaturated fatty acids and free radicals/reactive oxygen species. Studies have suggested that oxidative stress is an important pathogenic mechanism of diabetic complications caused by hyperglycemia, and the incidence of DR is associated with high oxidative stress in the body and the accompanying oxidative damage[10,11]. Biomarkers are biochemical indicators that can be used to mark changes in the structure or function of systems, organs, tissues, and cells. Effective biomarkers have been used for the diagnosis and classification of various diseases, including diabetes and diabetic microangiopathy. Serum malondialdehyde (MDA), 8-hydroxydehydroguanosine (8-OHdG), superoxide dismutase (SOD), and reduced glutathione (GSH) are common indicators of clinical oxidative stress. Currently, studies assessing the correlation between changes in blood oxygen saturation, oxidative stress markers, and risk of DR are limited. Therefore, our study mainly aimed to explore and discuss the changes in blood oxygen saturation and oxidative stress markers in patients with DR to reflect their condition to a certain extent, and for the early detection and treatment of patients, thus, improving the cure rate of patients.

MATERIALS AND METHODS

Data

A total of 94 patients (94 eyes) with DR (DR group) diagnosed at Jianyang people's Hospital between March 2019 and June 2020, and 100 volunteers (100 eyes) (control group) without eye diseases, were included in this study. The inclusion criteria comprised: patients (1) With DR aged 48–79 years; and (2) who met the diagnostic criteria of DR, as established by Practical Ophthalmology (3rd Edition)[12]. The exclusion criteria comprised: patients (1) With eye infection; (2) cataract; (3) glaucoma; (4) high myopia or hyperopia; (5) acute myocardial infarction; and (6) diseases of the blood system and autoimmune diseases. Based on the formation of retinal neovascularization, DR was divided into proliferative and non-proliferative DR.

Fundus vessel findings were confirmed by fundus fluorescein angiography (FFA). The control group consisted of volunteers with normal fundus and FFA findings.

The study bidding document and related materials were used after the medical ethics committee approved this study and decided the disease release. Informed consent was signed by patients and their families before the implementation of the study.

Measurement of oxygen saturation of retinal vessels

The OXYMAP T1 non-invasive retinal oxygen saturation analyzer was used to measure the retinal dynamic venous oxygen saturation of all participants before and after treatment. All the test operations were performed by the same technician; the test was repeated three times, and the average value was recorded.

Measurement of retinal blood vessel diameter

We administered tropicamide and waited for 5 min for pupil dilation, following which, an APS-B fundus color camera (Chongqing Kanghua Technology Co., Ltd.) was used to obtain 300-field fundus photos of the posterior pole of each eye. A 2.5-cm diameter circle was drawn with the center of the optic disc as the center. Two concentric circles (0.5- and 1.0-cm diameters, respectively) were then drawn from the edge of optic disc. The superior temporal and inferior retinal arteriovenous diameters intersecting the two concentric circles were measured twice at each measuring point.

Measurement of serum oxidative stress indicators

Serum MDA, 8-OHdG, SOD, and GSH levels were evaluated as follows: After maintaining 1 wk of glucose stability, a 5-mL fasting venous blood sample was collected from the patients with type 2 diabetes mellitus and from the control group on the next morning. Serum separation was performed at 3000 r/min. SOD level was measured using the xanthine oxidase method, MDA level was measured using the thiobarbital method, and GSH level was measured using the dithiobarbital colorimetric method. A competitive inhibition enzyme-linked immunosorbent assay was used to measure the 8-OHdG level. The kit was purchased from Trevigen (USA), and the procedure was performed strictly in accordance with the kit instructions.

Statistical analysis

The measurement indices of the patients in this study, such as retinal artery and retinal vein oxygen saturation, age, body mass index, and serum MDA and 8-OHdG levels, were in line with approximate normal distribution or normal distribution, based on the P-P plot and Q-Q plot, and expressed as mean

± SD. Enumeration data are expressed as percentage. The χ^2 test was used for sex comparison. Professional Statistical Package for the Social Sciences software version 21.0 software was used for data processing, and the test significance level was set at an α value of 0.05.

RESULTS

Baseline data of the study subjects

Baseline data, including age, sex, affected side distribution, hypertension, and diabetes in the DR and control groups were collected and analyzed; the two groups had good equilibrium and comparability ($P > 0.05$), and all patients in the two groups had good equilibrium (Table 1).

Comparison of retinal artery and venous oxygen saturation in the two groups

The retinal artery and venous oxygen saturation in the DR group were 95.70 ± 5.20% and 63.50 ± 4.41%, respectively, which were significantly higher than those in the control group (92.63 ± 4.50% and 60.83 ± 3.72%, respectively) (*t*-values, 4.405 and 4.568, respectively; $P < 0.05$) (Figure 1A).

Comparison of the retinal artery and vein diameters in the two groups

The diameters of the retinal arteries and veins were 12.06 ± 2.15 pixels and 15.83 ± 3.56 pixels in the DR group and 11.80 ± 2.07 pixels and 15.27 ± 3.30 pixels in the control group, respectively. The difference was not statistically significant (*t*-values, 0.858 and 1.137, respectively; $P > 0.05$) (Figure 1B).

Comparison of serum oxidative stress indicators between the two groups

Serum MDA and 8-OHdG levels in the DR group were significantly higher than those in the control group ($P < 0.05$). Compared with the control group, patients in the DR group had a significantly more aggravated oxidative stress injury (Table 2).

Comparison of retinal artery and venous oxygen saturation in patients with different diabetic retinopathy (DR) stages

In patients with proliferative DR, the retinal artery and venous oxygen saturation were 94.00 ± 4.95% and 61.80 ± 4.17%, respectively, which were significantly lower than those in patients with simple DR (98.21 ± 5.13% and 66.01 ± 4.28%, respectively) (*t*-values, −3.988 and −4.753, respectively; $P < 0.05$) (Figure 2A).

Comparison of the retinal artery and vein diameters in patients with different DR stages

The retinal artery and vein diameters were 12.22 ± 1.98 pixels and 16.17 ± 3.18 pixels, respectively, in the proliferative DR group, and 11.95 ± 2.04 pixels and 15.60 ± 3.38 pixels, respectively, in the simple DR group. The difference was not statistically significant (*t*-values, −0.637 and −0.822, respectively; $P > 0.05$) (Figure 2B).

Comparison of serum oxidative stress indices in patients with different DR stages

Serum MDA and 8-OHdG levels in patients with proliferative DR were significantly higher than those in patients with simple DR ($P < 0.05$). Serum SOD and GSH levels in patients with proliferative DR were significantly lower than those in patients with simple DR ($P < 0.05$) (Table 3).

Retinal color images of typical cases

The results of retinal color images of different subjects were shown in the Figure 3.

DISCUSSION

DR is the most common and severe blood vessel complication in diabetes, and its incidence has been increasing annually. Progression of DR from the non-proliferative stage to the proliferative stage can lead to irreversible visual damage, which is an important cause of blindness in the patient. The pathogenesis of DR is complex, and the main pathological changes include changes in the intraocular environment caused by abnormal blood vessel proliferation, resulting in retinal tissue hypoxic-ischemic injury[13,14].

Our study showed that the oxygen saturation of the retinal artery and retinal vein in the DR group was significantly higher than that in the control group. This can be explained by the reduced retinal oxygen consumption in DR. Considering these results, more in-depth and reliable studies on retinal oxygen consumption in DR should be conducted in the future. Furthermore, in this study, the retinal artery and retinal vein oxygen saturation of patients with proliferative DR was significantly higher than that of patients with simple DR. Some researchers[15-17] used a device similar to the one used in this

Table 1 The baseline data of the study subjects, *n* (%)

Group	DR group (*n* = 94)	Control group (*n* = 100)	t/χ^2 value	*P* value
Age (yr)	65.8 ± 7.0	64.4 ± 8.1	1.284	0.201
BMI (kg/m^2)	24.6 ± 1.9	24.4 ± 2.0	0.713	0.477
Sex			1.195	0.274
Male	51 (54.26)	62 (62.00)		
Female	43 (45.74)	38 (38.00)		
Affected side distribution			1.959	0.162
Left side	47 (50.00)	60 (60.00)		
Right	47 (50.00)	40 (40.00)		
Hypertension			2.561	0.110
Yes	18 (19.15)	29 (29.00)		
No	76 (80.85)	71 (71.00)		
Dyslipidemia			1.909	0.176
Yes	32 (34.04)	25 (25.00)		
No	62 (65.96)	75 (75.00)		

DR: Diabetic retinopathy; BMI: Body mass index.

Table 2 Comparison of serum oxidative stress indicators between the two groups of subjects (mean ± SD)

Group	DR group (*n* = 94)	Control group (*n* = 100)	*t* value	*P* value
MDA (μmol/L)	7.50 ± 1.50	3.82 ± 0.84	21.246	< 0.05
8-HdG (ng/mL)	107.50 ± 22.51	49.63 ± 8.11	24.102	< 0.05
SOD (U/L)	71.33 ± 14.80	93.64 ± 17.26	-9.637	< 0.05
GSH (mg/L)	163.81 ± 38.51	211.07 ± 25.46	-10.14	< 0.05

MDA: Serum malondialdehyde; 8-HdG: 8-hydroxydehydroguanosine; SOD: superoxide dismutase; GSH: Reduced glutathione.

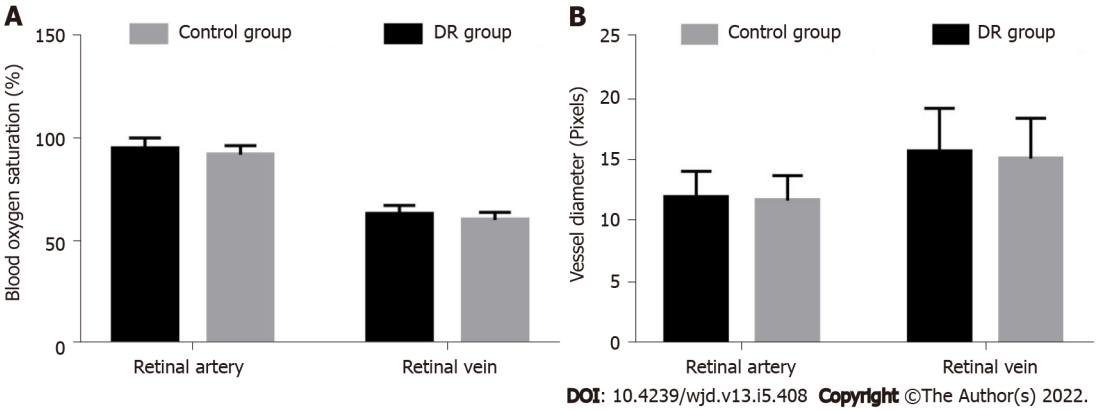
Table 3 Comparison of serum oxidative stress indexes in patients with different diabetic retinopathy stages (mean ± SD)

Disease classification	Simple (*n* = 56)	Proliferative (*n* = 38)	*t* value	*P* value
MDA (μmol/L)	6.11 ± 1.38	9.55 ± 1.43	-11.688	< 0.05
8-HdG (ng/mL)	90.53 ± 20.54	132.51 ± 19.82	-9.862	< 0.05
SOD (U/L)	83.02 ± 11.68	54.10 ± 13.01	11.249	< 0.05
GSH (mg/L)	187.46 ± 32.74	128.96 ± 26.74	9.135	< 0.05

MDA: Serum malondialdehyde; 8-HdG: 8-hydroxydehydroguanosine; SOD: superoxide dismutase; GSH: Reduced glutathione.

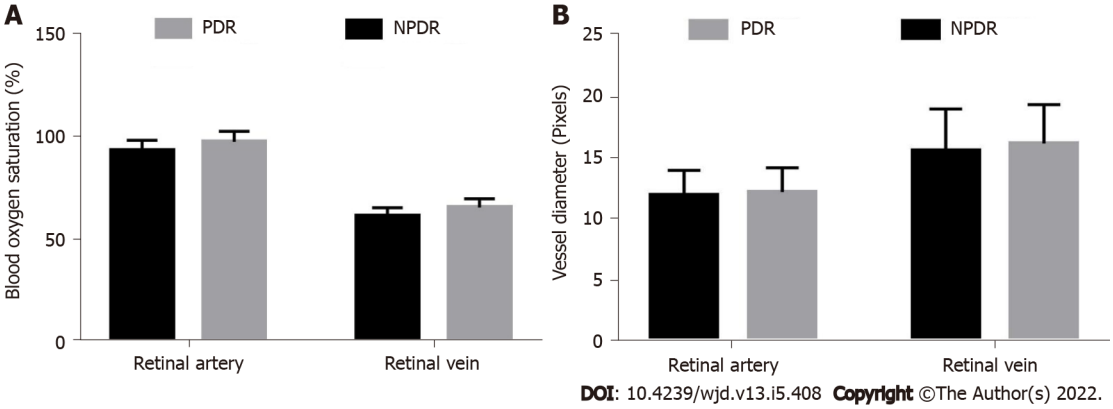
study to evaluate the main arteries and veins around the retinal optic disc and showed that blood oxygen saturation increased with the severity of DR, which was consistent with our results. Due to retinal hypoxic-ischemic injury, oxygen accumulates in retinal arteries and veins, and blood oxygen saturation increases, leading to insufficient retinal blood perfusion and oxygen supply. Simultaneously, oxygen free radicals and related cytokines in the body infiltrate the blood-retinal barrier, acting on retinal blood vessels, which also induces retinal tissue damage[18].

Some researchers[19-21] have used a variety of methods to measure the retinal vessel diameter in DR. In this study, there was no statistically significant difference in the diameter of the retinal artery and vein between the DR and control groups, or in the diameter of the retinal artery and vein between patients with proliferative DR and patients with simple DR. However, although the difference was not



DOI: 10.4239/wjd.v13.i5.408

Figure 1 Histograms of retinal artery, venous oxygen saturation, diameters of retinal arteries and veins of two groups of subjects. A: Histograms of retinal artery and venous oxygen saturation; B: Histograms of the diameters of retinal arteries and veins. DR: Diabetic retinopathy.



DOI: 10.4239/wjd.v13.i5.408

Figure 2 Histogram of retinal artery, venous oxygen saturation and vein diameters in patients with different diabetic retinopathy stages. A: Histogram of retinal artery and venous oxygen saturation; B: Histogram of retinal artery and vein diameters. PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy.

DOI: 10.4239/wjd.v13.i5.408

Figure 3 Results of retinal color images of different subjects. A: The control group; B: The simple diabetic retinopathy (DR) group; C: The proliferative DR group.

significant, it can be observed that the diameter of the retinal arteriovenous vessels in the DR group showed an upward trend compared with those in the control group, which is consistent with the results of most previous studies. However, the exact mechanism associated with the increase in retinal vessel diameter in DR remains unclear, which may be related to the obstruction of blood flow in small vessels caused by high blood glucose level and hypoxia. Moreover, retinal inflammation may also affect the diameter of the retinal arteries and veins.

Oxidative stress is not only an important factor in diabetes but also a key risk factor for DR. 8-OHdG is a biomarker for oxidative stress[22]. MDA is one of the final products of the peroxidation reaction between oxygen free radicals and unsaturated fatty acids in the cell membrane, which can indirectly reflect the content of oxygen free radicals and the degree of oxidative damage[23]. 8-OHdG and MDA are currently recognized as sensitive indicators for assessing oxidative stress. Due to their function and structure, nerve cells have higher oxygen supply requirements and are more sensitive to peroxide damage[24]. SOD and GSH are strong reducing agents in the body and can directly reduce oxidants, thus, reducing oxidative stress damage[25]. Therefore, the content of SOD and GSH can reflect the ability of the body to resist oxidative stress. The results of this study showed that the serum MDA and 8-OHdG levels in the DR group were significantly higher than those in the control group, and the serum SOD and GSH levels were significantly lower in the DR group than those in the control group, suggesting that the oxidative stress level of patients with DR increased with the increase in the degree of retinopathy. Therefore, oxidative stress may be involved in the incidence of DR, and high oxidative stress level may be a risk factor for the incidence of DR. Furthermore, the serum MDA and 8-OHdG levels in patients with proliferative DR were higher than those of patients with simple DR, and serum SOD and GSH levels were lower in patients with proliferative DR than those in patients with simple DR, indicating that oxidative stress increased significantly with the progression of the disease and retinopathy. Therefore, early simultaneous use of antioxidant stress and anti-inflammatory therapy may be more effective in delaying and treating DR.

Based on the findings of existing studies[26-30], this study compared serum oxidative stress and blood oxygen saturation indices of patients at different DR stages and simultaneously measured the retinal vessel diameter to explore the exact mechanism of oxidative stress-induced DR and to provide a valuable basis for the clinical diagnosis and treatment of patients with DR. However, it should be noted that the average age of the patients with DR in this study was relatively old, and our sample size was small. However, age, sex, and other factors have little impact on the retinal vascular oxygen saturation and retinal vessel diameter; therefore, we do not expect a large experimental deviation associated with these factors in our study.

CONCLUSION

In conclusion, increased blood oxygen saturation of retinal arteries and veins and increased oxidative stress damage in patients with DR may be associated with decreased retinal capillary permeability and arterial oxygen dispersion, and may reflect the patient's condition to a certain extent.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is the most important manifestation of diabetic micro-vasculopathy and is mainly related to the course of diabetes and the degree of blood glucose control, while the age of diabetes onset, sex, and type of diabetes have little influence on it.

Research motivation

This study explored the relationship between the changes of retinal arterial and venous oxygen saturation and oxidative stress injury and retinal capillary permeability and arterial oxygen diffusion, and whether it can reflect the patient's condition.

Research objectives

The study aimed to explore the effective monitoring index to effectively reflect the condition of patients with diabetic retinopathy.

Research methods

Total 94 patients (94 eyes) with DR (DR group) and 100 volunteers (100 eyes) (control group) without eye diseases, were included in this study. Arterial and venous blood oxygen saturation, retinal arteriovenous vessel diameter, and serum oxidative stress indicators in the two groups were compared. Based on the stage of the disease, the DR group was divided into the simple DR and proliferative DR groups for stratified analysis.

Research results

The oxygen saturation of the retinal vessels in the DR group was significantly higher than that in the control group. The retinal vessel diameters between the DR and control groups were not significantly different. The oxygen saturation of the retinal vessels in the patients with proliferative DR was

significantly higher than that in the patients with simple DR. The retinal vessel diameter in patients with proliferative DR was not significantly different from that of patients with simple DR.

Research conclusions

Increased blood oxygen saturation of retinal arteries and veins and increased oxidative stress damage in patients with DR may be associated with decreased retinal capillary permeability and arterial oxygen dispersion, possibly reflecting the patient's condition.

Research perspectives

Large sample studies should be performed in the further.

FOOTNOTES

Author contributions: Wang XL and Zhang M designed this retrospective study; Wang XL and Cai FR wrote the manuscript; Wang XL, Cai FR, Gao YX and Zhang J were responsible for sorting the data; and all authors approved the final version of the article.

Institutional review board statement: The study was approved by the Ethics Committee of Jianyang People's Hospital of Sichuan Province.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: We declare no competing interests.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

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World Journal of Diabetes

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World J Diabetes 2022 May 15; 13(5): 417-419

DOI: 10.4239/wjd.v13.i5.417

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Concomitant dysregulation of androgen secretion and dysfunction of adipose tissue induced insulin resistance

Marwan SM Al-Nimer

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Balbaa ME, Egypt; Ng HY, China; Xiong YP, China

Received: May 10, 2021
Peer-review started: May 10, 2021
First decision: September 5, 2021
Revised: September 7, 2021
Accepted: April 25, 2022
Article in press: April 25, 2022
Published online: May 15, 2022

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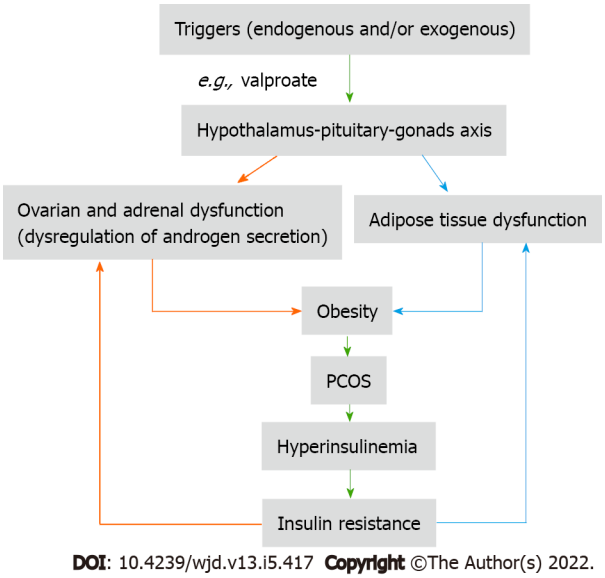
Abstract

Hyperandrogenism and hyperinsulinemia have resulted from dysfunction of the theca cell of the ovary and adipose tissue and each one potentiates the other in patients with androgen excess disorders *e.g.*, polycystic ovary disease and idiopathic hirsutism. Possible external and/or internal triggers can produce such cellular dysfunction. There is evidence that sodium valproate acts as a trigger of cellular dysfunction and produces both hyperinsulinemia and hyperandrogenism. Therefore, the elimination of these triggers can help the patients to recover from hyperinsulinemia, insulin resistance and hyperandrogenism.

Key Words: Hyperandrogenism; Hyperinsulinism; Central triggers; Polycystic ovary disease

Core Tip: There is a close relationship between hyperinsulinemia and androgen excess in patients with androgen excess disorders. These disorders result from the dysfunction of gonad and adipose cells under the influence of a specific trigger. Sodium valproate is an example of an external trigger that produces concomitant hyperinsulinemia and androgenism leading to polycystic ovary syndrome. Therefore, elimination of the triggers can lead to recovery from antiepileptic drugs while using insulin sensitizers and/or anti-androgens can help to solve this pathological problem.

Citation: Al-Nimer MS. Concomitant dysregulation of androgen secretion and dysfunction of adipose tissue induced insulin resistance. *World J Diabetes* 2022; 13(5): 417-419
URL: https://www.wjgnet.com/1948-9358/full/v13/i5/417.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i5.417



DOI: 10.4239/wjd.v13.i5.417

Figure 1 Interaction between hyperinsulinemia and hyperandrogenism as a result of an insult at the hypothalamic-pituitary-gonad axis.
PCOS: Polycystic ovary syndrome.

TO THE EDITOR

I read with great interest an elegant review by Unluhizarci *et al*[1] who presented the role of insulin in the androgen excess disorders (AEDs) taking polycystic ovary syndrome (PCOS) and idiopathic hirsutism as examples of AEDs. The authors filled the gap about the relationship between hyperandrogenism and hyperinsulinism and they highlighted the following important points: (1) The severity of insulin resistance is related to the phenotype of PCOS; (2) Hyperinsulinemia promotes the ovarian androgen synthesis in a mechanism not related to the gonadotropins; and (3) Using sodium valproate can cause androgen excess and hirsutism. Therefore, according to these important points, it is possible to consider that PCOS is a functional disease of concomitant dysregulation of androgen excess and dysfunction of the adipose tissue which is triggered by exogenous and/or endogenous insult at the hypothalamus-pituitary-target organs (gonads and adrenals)[2,3]. Some authors believe dysregulation of the androgen secretion in the theca cell of the ovary and adrenal gland can produce functional ovarian and adrenal hyperandrogenism, which not necessarily leads to hyperinsulinism and insulin resistance, while dysfunction of the adipose tissue can cause hyperinsulinism and insulin resistance[4]. Therefore, a question has arisen about which factor, trigger substance or event that causes the dysfunction of the theca cells and adipose tissue is still unknown.

So, any therapeutic intervention at the ovarian cell or adipose tissue will ultimately affect the other factor, because each factor potentiates the effect of another factor as Unluhizarci *et al*[1] mentioned in their review (Figure 1). Therefore, the use of insulin sensitizers and/or anti-androgens are of value in ameliorating the biochemical and clinical features of PCOS[5,6], but these medicines, when used as monotherapy, cannot correct hyperandrogenism and hyperinsulinemia at the same time.

Sodium valproate is a modifiable risk factor for the development of PCOS in epileptic and bipolar disorder women by increasing body weight and androgen production[7,8]. In addition, sodium valproate induces hyperinsulinism by having a direct effect on the beta-cell of the pancreas and an indirect effect by suppressing peripheral insulin-glucose uptake[9]. According to the valproate example, PCOS is the result of the vicious cycle (hyperinsulinism-hyperandrogenism) triggered by external or internal modifiable factors which are producing ovarian cell dysfunction. According to the literature, the triggers that cause dysfunction of the ovaries and adrenal glands act on the hypothalamic-pituitary-gonadal axis, and this explains why valproate can produce manifestations of PCOS in epileptic and bipolar depressed women. This effect seems to be gender-based because the relationship between insulin resistance and circulating androgens in obese young men is significantly inversed, while in PCOS women is significantly positive, indicating that there is a trigger factor that causes specific dysfunction of ovarian cells[10].

ACKNOWLEDGEMENTS

The author expressed his appreciation to the author of the article, Dr. Kursad Unluhizarci and his team, Department of Endocrinology, Erciyes University Medical School, Kayseri, Turkey, for doing this type

of research.

FOOTNOTES

Author contributions: Al-Nimer MS wrote and revised the letter.

Conflict-of-interest statement: The author declares no conflict of interest.

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S-Editor: Wang JJ
L-Editor: Filipodia
P-Editor: Wang JJ

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World Journal of Diabetes

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World J Diabetes 2022 May 15; 13(5): 420-421

DOI: 10.4239/wjd.v13.i5.420

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Admission hemoglobin level and prognosis of type 2 diabetes mellitus and possible confounding factors: Correspondence

Pathum Sookaromdee, Viroj Wiwanitkit

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ekine-Afolabi B, United Kingdom; Wan XH, China

Received: January 19, 2022
Peer-review started: January 19, 2022
First decision: March 11, 2022
Revised: March 12, 2022
Accepted: April 20, 2022
Article in press: April 20, 2022
Published online: May 15, 2022

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Abstract

This letter to editor discusses on the publication on admission hemoglobin level and prognosis of type 2 diabetes mellitus. A comment on published article is raised. The specific confounding conditions on the hemoglobin level are mentioned. Concerns on clinal application are raised and discussed.

Key Words: Diabetes; Hemoglobin; Confounding; Type 2 diabetes mellitus

Core Tip: This letter to editor discussing on the publication on admission hemoglobin level and prognosis of type 2 diabetes mellitus. Concerns on clinal application are raised and discussed.

Citation: Sookaromdee P, Wiwanitkit V. Admission hemoglobin level and prognosis of type 2 diabetes mellitus and possible confounding factors: Correspondence. *World J Diabetes* 2022; 13(5): 420-421
URL: https://www.wjgnet.com/1948-9358/full/v13/i5/420.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i5.420

TO THE EDITOR

We read with interest a case report on "Association between admission hemoglobin level and prognosis in patients with type 2 diabetes mellitus" by Song *et al*[1]. A

retrospective examination of patients diagnosed with type 2 diabetes mellitus (T2DM) bet was undertaken[1]. End-stage renal disease or a 50% drop in estimated glomerular filtration rate was the composite outcome[1]. Song *et al*[1] concluded that Hemoglobin levels and renal damage were found to have a U-shaped connection in T2DM patients. Hemoglobin levels below 13.3 g/dL at admission are an independent indicator of renal injury[1]. This report by Song *et al*[1] might add some data on application of hemoglobin level in monitoring of diabetic patient. In type 2 diabetes patients, Matsuoka *et al*[2] found that the duration of hypoglycemia was inversely associated with hemoglobin and hemoglobin A1C levels, and was longer at night than during the day. The kidney issue could be the result of a protracted period of hyperglycemia.

There are many possible confounding conditions on the hemoglobin level. In our setting in Indochina, many local people have a common inherited disorder, thalassemia, that has low hemoglobin level. In these thalassemic patients, renal impairment is also common regardless having diabetes or not [3]. Therefore, the conclusion on association by Song *et al*[1] might be applicable in some settings, but not all settings, such as our setting in Indochina. This correspondence can provide a novel insight that the application of hemoglobin level as an indicator might be limited in the area with high prevalence confounding hemoglobin disorder problem.

FOOTNOTES

Author contributions: Sookaromdee P gave ideas, analyzed the data, wrote the manuscript, revising and approving final submission; Wiwanitkit V gave ideas, analyzed the data, revising, supervising and approving final submission; All authors have read and approve the final manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com

ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 June 15; 13(6): 422-470

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJD* as 3.763; IF without journal self cites: 3.684; 5-year IF: 7.348; Journal Citation Indicator: 0.64; Ranking: 80 among 145 journals in endocrinology and metabolism; and Quartile category: Q3.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL

World Journal of Diabetes

ISSN

ISSN 1948-9358 (online)

LAUNCH DATE

June 15, 2010

FREQUENCY

Monthly

EDITORS-IN-CHIEF

Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS

https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE

June 15, 2022

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https://www.wjgnet.com/bpg/gerinfo/242

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World Journal of Diabetes

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World J Diabetes 2022 June 15; 13(6): 422-433

DOI: 10.4239/wjd.v13.i6.422

ISSN 1948-9358 (online)

EVIDENCE REVIEW

Impact of stopping smoking on metabolic parameters in diabetes mellitus: A scoping review

Magdalena Walicka, Cristina Russo, Michael Baxter, Isaac John, Grazia Caci, Riccardo Polosa

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Moreno-Gómez-Toledano R, Spain; Shuang WB, China
A-Editor: Yao QG, China

Received: December 20, 2021
Peer-review started: December 20, 2021
First decision: April 18, 2022
Revised: May 1, 2022
Accepted: May 21, 2022
Article in press: May 21, 2022
Published online: June 15, 2022

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Abstract

The purpose of this scoping review is to create a single narrative that describes the impact of smoking cessation on metabolic parameters in people with diabetes. It is generally well accepted that smoking enhances the harmful effects of elevated blood glucose levels, accelerating the vascular damage seen in patients with diabetes. Smoking cessation has clear benefits in terms of reducing cardiovascular morbidity and mortality. However, there is less evidence for the impact of smoking cessation on other diabetes-related complications. Studies in people with diabetes have shown improvement as well as temporary deterioration in glycemic control after ceasing smoking. Only a few studies have described the effect of quitting smoking on insulin resistance and lipid parameters, however, their results have been inconclusive. In this situation, healthcare professionals should

not assume that cessation of smoking will improve metabolic parameters in patients with diabetes. It seems they should, first of all, emphasize the prevention of weight gain that may be associated with quitting smoking. The lack of data regarding the metabolic effects of smoking and smoking cessation in diabetes is very disappointing and this area needs to be addressed.

Key Words: Smoking; Smoking cessation; Diabetes; Insulin resistance; Glucose; Lipids

Core Tip: Results of the studies regarding the impact of smoking cessation on metabolic parameters in patients with diabetes are inconsistent. Healthcare professionals should not assume that metabolic parameters in patients with diabetes who stop smoking will improve. It seems that the top priority after smoking cessation should be the prevention of weight gain. Further studies of the effects of quitting smoking on metabolic parameters among people with diabetes are required to provide an evidence base for healthcare advice to managed patients and to assist healthcare providers to implement the most effective interventions.

Citation: Walicka M, Russo C, Baxter M, John I, Caci G, Polosa R. Impact of stopping smoking on metabolic parameters in diabetes mellitus: A scoping review. *World J Diabetes* 2022; 13(6): 422-433
URL: https://www.wjgnet.com/1948-9358/full/v13/i6/422.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i6.422

INTRODUCTION

Approximately 1.3 billion people worldwide use tobacco, most commonly in the form of tobacco smoking, and more than 7 million people die every year as a result of smoking related conditions[1,2]. Smoking is the main cause of lung cancer, chronic obstructive pulmonary disease, and cardiovascular disease[3,4].

Exposure to cigarette smoke is associated with vascular damage, endothelial dysfunction, and activation of oxidative stress, inflammatory pathways, coagulation, and fibrinolysis[5,6]. A similar mechanism of endothelial dysfunction is described for people with diabetes. It is therefore not surprising that smoking enhances the combined harmful effects of elevated blood glucose levels, accelerating vascular damage in diabetic patients who smoke[7,8].

Smokers with diabetes [both type 1 diabetes (T1D) and type 2 diabetes (T2D)] may be at a higher risk due to the direct effect of vascular damage as well as the indirect adverse effect that smoking has on glycemic control and lipid levels[9].

The risk of cardiovascular events in diabetic patients is reduced with smoking cessation[10]. In the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation study, smoking cessation in those with diabetes was associated with a 30% reduction in all-cause mortality [11]. A comprehensive evaluation of predicted coronary heart disease (CHD) among current and ex-smokers who had T2D in Spain found that ex-smokers had approximately 20% lower CHD risk at 10 years compared to current smokers[12].

Although there is evidence that patients with diabetes can reduce the risk of macrovascular complications by giving up smoking, there is no conclusive evidence for the impact on the risk of microvascular complications[9,13,14]. The impact of quitting smoking on microvascular complications of diabetes and its metabolic indices is unclear. Furthermore, stopping smoking is known to cause weight gain which in turn may have unpredictable metabolic effect in patients with diabetes.

To the best of our knowledge, there have been no published systematic reviews to quantify the health benefits of smoking cessation in the diabetes population to date. The purpose of this scoping review is to create a single narrative describing the impact of smoking cessation in people with diabetes on glycemic control, insulin resistance and insulin secretion, and lipid abnormalities as well as biochemical parameters of nephropathy.

SEARCH METHODS

The published literature on the impact of stopping smoking on metabolic indices, including glycemic control, insulin resistance, and lipid abnormalities was systematically reviewed in September and October 2021. The studies on biochemical parameters of nephropathy were also included. The literature

search was conducted using the following databases: PubMed, Embase, ScienceDirect library, Database of Abstracts of Reviews of Effects, Scopus, and Google Scholar, using medical subject headings. We also used an artificial intelligence technology-based open multidisciplinary citation analysis database named Reference Citation Analysis. Search queries were developed by a trained librarian experienced in developing search strategies for reviews and were based on diabetes, smoking cessation, fasting plasma glucose (FPG) levels, hemoglobin A1c (HbA1c), insulin resistance, insulin secretion, lipids [total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoproteins (VLDL)], microalbuminuria, creatinine. More specifically, search terms included ("smoking cessation" OR "former smokers" OR "ex-smokers" OR "stop smoking" OR "quitting") AND ("diabetes") AND ("glucose" OR "glycemi*" OR "HbA1c" OR "insulin resistance" OR "HOMA" OR "insulin secretion" OR "total cholesterol" OR "HDL" OR "LDL" OR "VLDL" OR "microalbuminuria" OR "albuminuria" OR "creatinine" OR "GFR"). Search results were filtered to include only human studies and published from 1980. The titles, abstracts, and full texts of the search results were sequentially and independently screened by MW and GC for inclusion. A few studies were identified, including cross-sectional, case-control, and cohort studies, randomized clinical trials, and observational clinical studies, as well as systematic reviews and meta-analyses. The references of relevant studies were also manually reviewed for additional eligible citations.

SMOKING CESSATION AND INCIDENCE OF DIABETES

According to a meta-analysis conducted by Pan *et al*[15], recent quitters are at higher risk for developing diabetes, although this risk progressively declines with time[15].

It is often found that giving up smoking leads to a significant increase in weight[16,17]. According to a large prospective United Kingdom study, smoking abstenance was associated with an average weight gain of 8.79 kg at eight years, while continuing smokers gained only 2.24 kg[17]. This has been confirmed in a meta-analysis showing that quitting smoking is associated with a bodyweight gain of 4-5 kg after 12 mo of abstinence, with most of the weight gain occurring between the third and the sixth month after quitting[18]. As nicotine (in tobacco cigarettes) suppresses appetite and increases resting metabolic rate[19], people who stop smoking gain weight because they have diminished resting energy expenditure and increased appetite. Moreover, quitters often substitute smoking with excessive eating/snacking, as shown in several studies of eating behaviors[20].

It is likely that the weight gain associated with stopping smoking is responsible for the initial increase in risk of developing T2D. The increase in the risk of T2D after quitting was directly proportional to weight gain, but not increased among quitters without weight gain[21].

SMOKING CESSATION AND GLYCAEMIC CONTROL

Patients with diabetes can become more insulin resistant with worsening glycemic control when they gain weight. Pani *et al*[22], examining the predictors of diabetes progression (defined as HbA1c^3 7% or the initiation of hypoglycemic therapy), found that weight gain was an independent predictor. Each extra pound of weight that is gained increases the risk of developing diabetes by 2%.

In patients with diabetes, quitting smoking may cause increased appetite, caloric intake, and weight gain, which would predictably lead to the worsening of glycemic control. In contrast, stopping smoking appears to have a beneficial effect on carbohydrate metabolism in the long run which may potentially mitigate the initial adverse metabolic effects of smoking cessation[23].

Considering the complex interplay among factors that affect glycemic control, some uncertainty in the findings of studies that investigate the impact of stopping smoking on glycemic control might be anticipated. Studies comparing smokers to ex-smokers, both with T1D and T2D, demonstrated that active smoking is associated with worse glycemic control. In the study of Dinardo *et al*[24] current smokers (with an average smoking history of 30 years, an average daily habit of one pack of cigarettes *per* day) had higher mean HbA1c in comparison with former smokers. In the multiple linear regression analysis, current smoking was independently and significantly associated with higher HbA1c. Braffett *et al*[14], using the data of a well-characterized cohort group with T1D from the Diabetes Control and Complications Trial (1983-1993), showed that in comparison to former smokers (subjects who previously smoked but quit > 3 mo prior to baseline), current smokers (subjects who currently smoked or quit < 3 mo prior to baseline) had higher mean HbA1c levels (average difference of 0.31%) over an average of 6.5 years of follow-up. The mean HbA1c levels for former smokers were similar to those of whom have never smoked. In relation to not only the current smoking status but also to its lifetime intensity and duration, the mean HbA1c levels were higher (average difference 0.22%) for current smokers with more than 10 pack-years in comparison to former smokers with less than 10 pack-years.

In an observational study of 10692 adult smokers with T2D, 29% of patients who had quit smoking and remained abstinent for at least 1 year revealed an increase in HbA1c of 0.21% with the need to intensify glucose-lowering treatment[25]. In further observation, HbA1c level decreased as abstinence

continued, and became comparable to this in people who continued to smoke after a 3-year follow-up. Patients who stopped smoking gained weight (4.68 kg on average), but the results suggested that the change in weight was not directly related to the increase in HbA1c.

In Asiatic patients quitting smoking is generally associated with an improvement in glycemic control. In a study of 2490 male Japanese patients with T2D, HbA1c decreased linearly with the years after stopping smoking; however, there was no correlation with FPG[23]. Similarly, in a study of 7763 Chinese men with T2D, the HbA1c level decreased progressively with each year that the patients had stopped smoking; in this study, FPG levels decreased[26]. In a smaller retrospective cohort study comprising 241 Taiwanese patients with T2D, the group completing the smoking cessation program showed a significant decrease in FPG and HbA1c levels at 3-mo follow-up compared to baseline. Due to the fact that the analyses of cardiometabolic factors were carried out before and after participation in the smoking cessation program in the whole group (regardless of the outcome of the smoking cessation program), it is difficult to interpret these results[27].

In contrast, there are a number of studies on Asian patients failing to show improvement in glycemic control after stopping smoking. In a randomized controlled trial conducted in China, results of quitting smoking did not affect HbA1c levels at 1-year follow-up. The study included 557 smokers with T2D[28].

In the meta-analysis published by Kar *et al*[29] there was no statistically significant difference in HbA1c between smokers and quitters. However, when the meta-analysis was reanalyzed including studies comparing nonsmokers and active smokers, a statistically significant difference was demonstrated and this was positively associated with smoking duration; increasing as the years of smoking increased.

A summary of the studies evaluating smoking cessation's effect on HbA1c is shown in Table 1. The table also includes HbA1c data from studies of smokers with diabetic nephropathy.

SMOKING CESSATION AND INSULIN RESISTANCE AND INSULIN SECRETION

The pathogenic mechanisms underlying T2D are a balance between insulin resistance and beta-cell dysfunction. Smoking has been shown to influence both insulin resistance and insulin secretion. Studies on animals have shown that cigarette smoke can impair insulin production and secretion in addition to reducing beta-cell viability and proliferation[30].

There has also been speculation that nicotine in tobacco smoke could play a significant role in promoting insulin resistance. Although chronic exposure to nicotine may be necessary to impact insulin sensitivity in nicotine naive subjects, acute exposure to nicotine can cause negative effects on insulin sensitivity in individuals with pre-existing insulin resistance[31-33].

However, the direct effect of nicotine on insulin resistance is not supported in studies looking at the use of snus. Snus is an oral tobacco product that delivers significant levels of nicotine without producing any toxic combustion byproducts[34]. Since the 1980s, snus consumption has been growing in popularity in Sweden, gradually replacing cigarette smoking[35,36].

With the exception of one study, which has methodological issues including a flawed cross-sectional design and the lack of adjustment for smoking history in snus users[37], there is clear evidence that snus use does not produce a significant rise in diabetes risk[38-41]. Moreover, there was no association between snus use and insulin levels or glucose tolerance in a large study involving 1266 subjects and primarily focused on cardiovascular risk factors[42]. Insignificant relative risks for T2D were reported in a meta-analysis for never-smoking current, former and ever-snus users[43]. In addition, impaired glucose tolerance and related endpoints were not associated in any significant way.

It has been demonstrated that smokers have greater waist-to-hip circumference ratios[44,45]. Waist-to-hip circumference ratio is one of the most pragmatic clinical measures of central obesity. One of the major contributing factors in obesity-related metabolic complications is fat distribution. The visceral abdominal depot (abdominal obesity) is linked to metabolic dysfunction (cardiovascular disease, insulin resistance, T2D). Conversely, lower body adiposity (gluteofemoral obesity) is associated with improved cardiovascular and metabolic profiles[46]. The abdomen adipose tissue is characterized by the rapid uptake of diet-derived fat and a high lipid turnover that is easily stimulated by stress hormones[46]. Increased release of free fatty acids and abnormalities in adipokine secretion observed in people with abdominal obesity promote insulin resistance[47].

Compared with nonsmokers, smokers are characterized by greater insulin resistance and hyperinsulinemia[48]. However, little research has been conducted on the impact of smoking cessation on insulin resistance and insulin secretion.

Smoking cessation may be associated with worsening fat distribution. In a population-based study (Inter99 Study) performed in Copenhagen, the mean increase in waist circumference after quitting at the one-year follow-up was 3.88 cm (42% of the quitters had increased their waist circumference by ≥ 5 cm). Quitters with high baseline tobacco consumption were more likely to have substantially increased waist circumference. In this study, abstinence from smoking was the most important predictor of substantial weight gain and a substantial increase in waist circumference[49]. Likewise, a study with the use of computed tomography showed that both current and former smoking is associated with increased

Table 1 The results of studies evaluating the effect of smoking cessation on hemoglobin A1c in diabetic patients

Ref.	Country/region	Study population	Study design	Effect
Dinardo *et al* [24], 2019	United States	T2D, *n* = 282	Cross-sectional, comparison of current smokers *vs* former smokers and never-smokers	Positive
Braffett *et al*[14], 2019	Multicenter (United States and Canada)	T1D, *n* = 1441	Retrospective analysis of the prospective cohort study, comparison of current smokers *vs* former smokers and never-smokers	Positive
Lycett *et al*[17], 2011	United Kingdom	T2D, *n* = 10692	Retrospective cohort study, observation of HbA1c in three groups: Continual smokers, long-term quitters, and relapsers	Negative
Ohkuma *et al* [23], 2015	Japan	T2D, *n* = 2490	Cross-sectional study, comparison of current smokers and former smokers *vs* never-smokers	Positive
Su *et al*[26], 2017	China	T2D, *n* = 7763	Cross-sectional study, comparison of current smokers, former smokers and never-smokers	Positive
Li *et al*[28], 2017	Hong Kong of China	T2D, *n* = 557	A randomized controlled trial, comparison of level of HbA1c and changes from baseline to 12-mo between quitters and non-quitters	Neutral
Kar *et al*[29], 2016	United States, Japan	T1D + T2D, *n* = 13719	Metanalysis, comparison of current smokers *vs* quitters	Neutral
Voulgari *et al* [57], 2011	Greece	T2D, *n* = 193	Prospective study, comparison of smokers *vs* former-smokers	Positive
Feodoroff *et al* [69], 2016	Finland	T1D, *n* = 3613	Prospective study, comparison of smokers *vs* non-smokers and former-smokers *vs* non-smokers	Positive
Reynolds *et al* [68], 2011	United States	T1D, *n* = 2124	Cross-sectional analysis of population-based study, comparison of current smokers *vs* non-smokers and former-smokers *vs* non-smokers	Neutral
Reynolds *et al* [68], 2011	United States	T2D, *n* = 348	Cross-sectional study, comparison of current smokers *vs* non-smokers and former-smokers *vs* non-smokers	Neutral

T1D: Type 1 diabetes; T2D: Type 2 diabetes; HbA1c: Hemoglobin A1c;

visceral adipose tissue[50]. In a population-based study performed in Norway, former smokers compared with current smokers had a lower waist to hip ratio (additionally among women, waist circumference was lower)[51].

However, studies assessing insulin resistance indicators in quitters do not have consistent results. There was a statistically significant negative correlation between homeostasis model assessment-estimated insulin resistance (HOMA-IR) values among asymptomatic, Korean male ex-smokers without diabetes[52]. In contrast, other studies (also in the groups without diabetes) showed that quitting smoking was associated with greater insulin resistance as measured by Quicki or HOMA-IR[53,54].

The observed discordance amongst the insulin sensitivity findings is likely to be due to a change in body weight. After stopping smoking, insulin sensitivity is likely to change because of weight fluctuations. It was shown that the HOMA-IR index after quitting significantly increases in weight gainers, but not in weight maintainers[55]. In the study by Heggen *et al*[56], no differences were found in HOMA-IR between quitters and smokers but the findings must be interpreted within the context of similar modest body weight changes in quitters and smokers at 3-mo follow-up.

These studies have most commonly included people without diabetes. The only study investigating the relationship between insulin resistance and smoking cessation among patients with diabetes is that of Ohkuma *et al*[23]. The authors found that smoking cessation has a time-dependent link with insulin resistance in Japanese patients with T2D; HOMA-IR levels decreased in ex-smokers over time relative to current smokers. HOMA-IR was also assessed in the prospective study, evaluating the effect of smoking on the progression of microalbuminuria in T2D. Smoking cessation was associated with the amelioration of insulin resistance parameters in spite of the small but significant increase in body mass index. This observation may be explained by the fact that many quitters increased their physical activity [57].

A summary of the studies evaluating smoking cessation's effect on HOMA-IR is shown in Table 2.

The search for publications on quitting and insulin secretion in patients with diabetes was unproductive. In the population without diabetes, Morimoto *et al*[58] found that the risk of impaired insulin secretion in an ex-smoker is similar to that in never-smokers, where the risk is almost twice as high in current smokers when compared with never smokers, with the magnitude of this increase being dose-dependent (*i.e.* increasing with a number of pack-years). Stadler *et al*[54] showed a 31% increase in beta-cell secretion (as measured by insulinogenic index 140) after > 3 mo of not smoking.

Table 2 The results of studies evaluating the effect of smoking cessation on homeostasis model assessment-estimated insulin resistance in diabetic patients

Ref.	Country	Study population	Study design	Effect
Ohkuma *et al*[23], 2015	Japan	T2D, *n* = 2490	Cross-sectional study, comparison of current smokers and former smokers *vs* never-smokers	Positive
Voulgari *et al*[57], 2011	Greece	T2D, *n* = 193	Prospective study, comparison of smokers *vs* former-smokers	Positive

T1D: Type 1 diabetes; T2D: Type 2 diabetes.

SMOKING CESSATION AND LIPIDS ABNORMALITIES

Patients with T2D characteristically have abnormal plasma lipids profiles which are marked by hypertriglyceridemia, reduced HDL cholesterol levels, and increased concentration of small dense LDL. These abnormalities are a result of a multifactorial process, including abdominal obesity, insulin resistance, increased free fatty acid flux, and inflammation[59]. Cigarette smoke has been shown to increase the atherogenic nature of the lipid profile[60]. Smoking is associated with increased triglycerides (TG), total cholesterol, and LDL, as well as reduced levels of cardioprotective HDL[61]. In a prospective study of 808 young Asian adults, smokers were three times more likely to have low HDL cholesterol and were 2.6 times more likely to develop hypertriglyceridemia[62]. There is a clear assumption in healthcare messaging that stopping smoking may correct dyslipidemia, which is especially relevant in smokers with diabetes. Studies, conducted on patients without diabetes, indicate that quitting smoking increases HDL levels[63,64]. The increase in HDL has frequently been observed in spite of weight gain experienced after cessation of smoking[63]. Evidence also indicates that smokers may have improved HDL function (increased cholesterol efflux capacity and decreased HDL inflammatory index) after quitting smoking[65].

Data on TG levels are conflicting. Some studies performed in the group without diabetes showed that smoking cessation is associated with a reduction of this lipid fraction[66], however, others studies have failed to confirm this[64].

Data on LDL is also limited, but evidence seems to suggest that smoking cessation does not affect LDL levels or LDL size[63,67].

A few studies have tested diabetic patients' lipid profiles after quitting smoking. Results are inconsistent. In Reynolds *et al*[68], 3466 youth who had T1D (*n* = 2887) or T2D (*n* = 579) and were smokers were examined for prevalence of tobacco use and the coexistence of cardiovascular risk factors. Compared to patients who were non-smokers, past smokers with T1D had significantly higher odds of having high LDL cholesterol levels, and those who were current smokers had significantly higher chances of having high TG levels. Patients with T2D did not exhibit these relationships, but the smaller numbers of patients included in the study could have influenced the statistical significance of the results.

In the study of Luque-Ramírez *et al*[12] patients with T2D who smoke had lower HDL and higher TG levels compared to their nonsmoking counterparts.

Lipid parameters were examined in two studies in patients with diabetic nephropathy. After stopping smoking for at least 1 year, patients had significantly lower total cholesterol, LDL, and HDL levels than those who continued to smoke[57]. In a similar study of patients with T1D, total cholesterol, TG, and LDL levels of current and former smokers were higher than those of non-smokers, whereas lower HDL levels were observed in current smokers[69].

A summary of the studies evaluating smoking cessation's effect on lipid parameters is shown in Table 3.

SMOKING CESSATION AND BIOCHEMICAL PARAMETERS OF NEPHROPATHY

It is well known that chronic kidney disease (CKD) and end-stage renal disease (ESRD) can complicate diabetes mellitus. Diabetic nephropathy is characterized by proteinuria and/or the decline of renal function [*e.g.* reduced glomerular filtration rate (GFR)][70]. Aside from high blood sugar levels, other risk factors that contribute to the development and progression of diabetic kidney disease include high blood pressure, dyslipidemia, and genetic predisposition[71]. Smoking may also be a factor in the development and progression of kidney failure possibly through a mechanism of progressive arteriolar damage, increased renovascular resistance, and increased intraglomerular capillary pressure[72-75]. While many studies have examined the relationship between cigarette smoking and kidney disease with conflicting results, a meta-analysis of 15 prospective cohort studies with 65064 incident cases of CKD suggests that smoking is as an independent risk factor in the general population[76].

Table 3 The results of studies evaluating the effect of smoking cessation on lipid parameters in diabetic patients

Ref.	Country	Study population	Study design	Effect
Reynolds *et al*[68], 2011	United States	T1D, *n* = 2124	Cross-sectional analysis of population-based study, comparison of current smokers *vs* non-smokers and former-smokers *vs* non-smokers	LDL–negative; HDL–neutral; TG–positive
Reynolds *et al*[68], 2011	United States	T2D, *n* = 348	Cross-sectional study, comparison of current smokers *vs* non-smokers and former-smokers *vs* non-smokers	Neutral
Luque-Ramírez *et al*[12], 2018	Spain	T2D, *n* = 890	Cross-sectional, observational study, comparison of smokers *vs* former-smokers	LDL–neutral; HDL–positive; TG–positive
Voulgari *et al*[57], 2011	Greece	T2D, *n* = 193	Prospective study, comparison of smokers *vs* former-smokers	LDL–positive; HDL–positive; TG–positive
Feodoroff *et al*[69], 2016	Finland	T1D, *n* = 3613	Prospective study, comparison of smokers *vs* non-smokers and former-smokers *vs* non-smokers	LDL–neutral; HDL–positive; TG–neutral

T1D: Type 1 diabetes; T2D: Type 2 diabetes; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TG: Triglycerides.

There is no clear impact of stopping smoking on characteristics associated with diabetic nephropathy. The effect of smoking cessation on microalbuminuria was investigated by Voulgari *et al*[57] in subjects with newly diagnosed T2D mellitus. Within a year of stopping smoking, the prevalence of those with microalbuminuria markedly declined compared to those who continued smoking. However, eGFR was comparable between the two study groups. Smokers who stopped smoking had a lower microalbuminuria rate irrespective of the effect of drug therapy (antihypertensive, hypolipidemic, and antidiabetic).

In a study of 2770 patients with T2D, Ohkuma *et al*[77] investigated the association of smoking and its abstinence with parameters of CKD. In comparison to non-smokers, former and current smokers had higher urinary albumin-creatinine ratios. In the former smokers, this ratio decreases linearly with increasing years after quitting smoking. Furthermore, current smokers' risk is related to how many cigarettes *per* day they smoke. After quitting smoking, age-adjusted creatinine-based GFR declined compared to the never-smokers but increased in parallel with increasing cigarette consumption. The increased eGFR of smokers may be related to glomerular hyperfiltration which is implicated as a mechanism for the progression of diabetic nephropathy[78].

Progressive kidney damage can result from glomerular hyperfiltration over time. According to Ohkuma's study, the proportion of smokers with CKD increased with the number of cigarettes they smoked *per* day (compared with never-smokers). However, as the years passed since quitting, the proportion of patients with CKD decreased. There was a significant increased HbA1c level for current smokers and a greater proportion of hypertension for ex-smokers compared with never smokers and current smokers with respect to the other risk factors for nephropathy in this study[77].

Using data from the Finnish Diabetic Nephropathy Study, which included 3613 T1D patients, the 12-year cumulative risk of microalbuminuria, macroalbuminuria, and ESRD by smoking status was calculated. Current and former smokers were more likely to have micro- and macro-albuminuria (ESRD for current smokers only) than non-smokers. There were no statistically significant differences in the 12-year cumulative risk of microalbuminuria and macroalbuminuria between former smokers and never smokers. There were significantly poorer glycemic control and lipid parameters for smokers compared to nonsmokers. Adjusting for HbA1c and lipid variables, the increased risk of diabetic nephropathy progression among current and former smokers was attenuated. Smoking-related changes in lipids and glucose control may account for the majority of nephropathic changes due to diabetes[69]. This observation suggests that poor glucose control and lipid alterations in smokers are the main drivers of nephropathic changes in diabetes.

A summary of the studies evaluating smoking cessation's effect on characteristics associated with diabetic nephropathy is shown in Table 4.

CONCLUSION

In addition to reducing overall and cardiovascular mortality, stopping smoking may provide significant additional health benefits to people with diabetes. It is important to note, however, that weight gain experienced after stopping smoking may attenuate some of these health benefits[79].

When considering the potential impact of stopping smoking on metabolic parameters in patients with diabetes, the benefits of cessation of smoking are less clear because the expected outcomes have not been consistently demonstrated. Studies have shown both improvements and temporary deterioration in glycemic control after quitting smoking. Only a few available studies have investigated the effect of

Table 4 The results of studies evaluating the effect of smoking cessation on biochemical parameters of nephropathy in diabetic patients

Ref.	Country	Study population	Study design	Effect
Ohkuma *et al* [77], 2016	Japan	T2D, *n* = 2770	Cross-sectional study, comparison of smokers, former-smokers and never-smokers	UACR–positive with increasing years after quitting
Voulgari *et al* [57], 2011	Greece	T2D, *n* = 193	Prospective study, comparison of smokers *vs* former-smokers	Microalbuminuria–positive
Feodoroff *et al* [69], 2016	Finland	T1D, *n* = 3613	Prospective study, comparison of smokers *vs* non-smokersand former-smokers *vs* non-smokers	Micro- and macroalbuminuria–positive

T1D: Type 1 diabetes; T2D: Type 2 diabetes; UACR: Albumin-creatinine ratio.

quitting smoking on insulin resistance and lipid parameters in diabetic patients. These studies also report inconsistent results. Smoking cessation appears to have a clear beneficial effect on markers of nephropathy, particularly after longer periods of smoking abstinence.

The review of the published literature found only a few studies, many of which had design and methodological shortcomings, and as such-need to be interpreted with caution. A major issue for this area of study is the lack of randomized controlled trials that have been carried out to date.

In an era of evidence-based medicine, the lack of data regarding the metabolic effects of smoking and smoking cessation in diabetes is very disappointing and needs to be addressed. Diabetes is one of the major population health issues, the consequence of which appears to be amplified by smoking. The lack of good quality research on the impact of smoking cessation on metabolic parameters in this population hampers clinicians' ability to give informed advice on the effectiveness and management of stopping smoking. This is a complex medical and sociological issue that demands a greater research focus to better inform people with diabetes and assist healthcare providers to implement the most effective interventions.

ACKNOWLEDGEMENTS

The authors would like to thank Ms. Erika Anastasi for her help with the literature search. The authors' thoughts and prayers are with all the victims of the Russian-Ukrainian conflict.

FOOTNOTES

Author contributions: Walicka M contributed to the conceptualization, literature search, and screening, writing, review, editing; Russo C, Baxter M and John I contributed to the writing, reviewing, editing; Caci G performed the literature search and screening, writing, reviewing, editing; Polosa R contributed to the conceptualization, writing, reviewing, editing, revising, supervising; all author read and approved the final version of the manuscript.

Supported by Department of Clinical and Experimental Medicine of the University of Catania, No. 6C813202024/1_3_02_07_01/2020.

Conflict-of-interest statement: RP is full tenure professor of Internal Medicine at the University of Catania (Italy). RP has received lecture fees and research funding from Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, MSD, Boehringer Ingelheim, Novartis, Duska Therapeutics, and Forest Laboratories. RP has also received grants from European Commission initiatives (U-BIOPRED and AIRPROM) and from the Integral Rheumatology & Immunology Specialists Network (IRIS) initiative. He has also served as a consultant for Pfizer, Global Health Alliance for treatment of tobacco dependence, CV Therapeutics, Boehringer Ingelheim, Novartis, Duska Therapeutics, ECITA (Electronic Cigarette Industry Trade Association, in the UK), Arbi Group Srl., Health Diplomats, and Sermo Inc. RP has served on the Scientific Advisory Board of Cordex Pharma, Inc., CV Therapeutics, Duska Therapeutics Inc, Pfizer, and PharmaCielo. RP is also founder of the Center for Tobacco prevention and treatment (CPCT) at the University of Catania and of the Center of Excellence for the acceleration of HArm Reduction (CoEHAR) at the same University, which has received support from FSFW to conduct 8 independent investigator-initiated research projects on harm reduction. RP has filed a patent application concerning an app tracker for smoking behaviour developed for ECLAT Srl. RP is involved in the following pro bono activities: scientific advisor for LIAF, Lega Italiana Anti Fumo (Italian acronym for Italian Anti-Smoking League), the Consumer Advocates for Smoke-free Alternatives (CASAA) and the International Network of Nicotine Consumers Organizations (INNCO); Chair of the European Technical Committee for standardization on "Requirements and test methods for emissions of

electronic cigarettes" (CEN/TC 437; WG4). All other authors have no declared relevant conflict of interest to declare in relation to this study.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 June 15; 13(6): 434-441

DOI: 10.4239/wjd.v13.i6.434

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Investigating the specificity of endothelin-traps as a potential therapeutic tool for endothelin-1 related disorders

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Specialty type: Pharmacology and pharmacy

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ahmed AA, Egypt; He Z, China
A-Editor: Zhu JQ, China

Received: January 27, 2022
Peer-review started: January 27, 2022
First decision: April 18, 2022
Revised: April 24, 2022
Accepted: May 28, 2022
Article in press: May 28, 2022
Published online: June 15, 2022

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Abstract

BACKGROUND

Endothelin (ET)-traps are Fc-fusion proteins with a design based on the physiological receptors of ET-1. Previous work has shown that use of the selected ET-traps potently and significantly reduces different markers of diabetes pathology back to normal, non-disease levels.

AIM

To demonstrate the selected ET-traps potently and significantly bind to ET-1.

METHODS

We performed phage display experiments to test different constructs of ET-traps, and conducted bio-layer interferometry binding assays to verify that the selected ET-traps bind specifically to ET-1 and display binding affinity in the double-digit picomolar range (an average of 73.8 rM, $n = 6$).

RESULTS

These experiments have confirmed our choice of the final ET-traps and provided proof-of-concept for the potential use of constructs as effective biologics for diseases associated with pathologically elevated ET-1.

CONCLUSION

There is increased need for such therapeutics as they could help save millions of lives around the world.

Key Words: Endothelin-1; Endothelin-traps; Diabetes; Heart failure; Chronic kidney disease; Novel therapeutic

Core Tip: This study verified the specificity of endothelin (ET)-traps, which are an Fc-based fusion protein that acts as a potential therapeutic for various cET-1 related disorders, including diabetes and chronic kidney disease. ET-traps, unlike ET receptor antagonists, do not completely block the ET system and hence have minimal side effects. ET-traps would help save millions of lives around the world.

Citation: Jain A, Bozovicar K, Mehrotra V, Bratkovic T, Johnson MH, Jha I. Investigating the specificity of endothelin-traps as a potential therapeutic tool for endothelin-1 related disorders. *World J Diabetes* 2022; 13(6): 434-441

URL: https://www.wjgnet.com/1948-9358/full/v13/i6/434.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i6.434

INTRODUCTION

Endothelin-1 (ET-1) is a vasoactive peptide synthesized and secreted by a diverse range of cells, and thus implicated in signaling events in a wide variety of target tissues[1]. ET-1 plays a key role in physiological functions. However, supraphysiological levels of ET-1 induce pathology and are implicated in a host of different diseases, including cardiovascular disease[2-4], neurodegenerative disorders[5-8], chronic kidney disease, different cancers, such as prostate cancer[1,9,10], pregnancy disorders like preeclampsia[7,11], as well as diabetes[8,12,13]. Given that a key feature of these diseases is elevated ET-1 Levels, one proposed strategy of therapeutic intervention is to target the increased levels of ET-1. To this end, we have created ET-traps, molecular constructs that bind and sequester increased levels of endogenous ET-1.

Diabetes is a serious metabolic complication that affects about more than 7% of the world population [14]. An increase in different extracellular matrix (ECM) proteins has been found to be a key pathological factor of diabetes[15,16]. The study by Jain *et al*[12] found an increase in collagen 4α1 and fibronectin both at the mRNA and protein levels. This increase was found in heart and kidney tissues and was found to be ET-1 dependent[12]. In addition, the increase in ECM proteins due to high glucose levels was found to be mediated *via* ET-1[17]**.** ET-1 Levels are in fact increased in patients with diabetes compared with control subjects[18,19]. Accordingly, our previous *in vitro* work confirmed the ET-traps to have an efficacious effect on cells treated with a pathological dose of ET-1, as well as those treated with pathologically high glucose (25 mmol/L)[12]. We also established the proof-of-concept (PoC) for ET-traps as a therapeutic in the diabetes disease space at the *in vivo* level. The use of ET-traps gave a significant reduction in different markers of diabetes disease pathology, which suggested the ET-traps could be considered a therapeutic for diabetes with a novel mechanism of action. Importantly, the ET-traps were found to be non-toxic at the proposed therapeutic concentration both *in vitro* and *in vivo*[12, 13].

ET-1

ET-1 exerts its effects by binding to the endothelin A and B receptors, two highly homologous cell-surface proteins that belong to the G-protein-coupled receptor superfamily[20]. The two receptors share about 60% similarity at the level of primary structure[1], *i.e.* both receptors exhibit a high polypeptide sequence identity with each other. Nevertheless, the two receptors show a clear distinction in ligand binding selectivity based on their ligand-binding domains.

Orry *et al*[21] constructed a model of interaction of the ET-1 peptide with the endothelin A receptor, where ET-1 makes contacts with both the N-terminal receptor domain and two different extracellular loops (ECL).

Further, amino acids of the C-terminal and residues in the third intracellular loop are important for ET-1 binding[22]. In this study, we performed binding affinity experiments to ascertain that our selected ET-traps bind specifically just to ET-1.

Homologs of ET-1

The ETs are a family of potent vasoactive peptides. ET-1 has two paralogs in the ET family; ET-2 and ET-3[23]. ET-1 was identified by Yanagisawa *et al*[24] in 1988. A year later, 2 homologs of ET-1 were discovered; ET-2 and ET-3[25].

ET-2 is a peptide encoded by the *EDN2* gene located on chromosome 1 in humans[26]. ET-2 has a key role in ovarian physiology[25]. Previous research findings have also revealed that ET-2 is critical for the growth and survival of postnatal mice and plays important roles in energy homeostasis, thermoregulation, and the maintenance of lung function[26].

ET-3 is a peptide that in humans is encoded by the *EDN3* gene[27]. The active peptide is a ligand for ET receptor type B (EDNRB). The interaction of this ET with EDNRB is essential for development of neural crest-derived cell lineages, such as melanocytes and enteric neurons[28].

Therefore, both the ET-1 paralogs (ET-2 and ET-3) are essential for different physiological processes and so it is important that any ET-1 sequestering agent selectively targets ET-1 and hence the problems associated with increased expression of ET-1 to avoid disrupting the remaining processes of the ET system.

In this study, we first performed phage display experiments to ascertain the binding of the ET-traps to ET-1. Phage display is one of the most powerful and widely used laboratory techniques for the study of protein–protein, protein–peptide and protein–DNA interactions[29]. This technology is based on expressing the protein or peptide of interest on bacterial virus protein coat, allowing the study of molecular interaction between the virion-displayed ligand (in this case ET-traps) and an immobilized target (*i.e.* ET-1).

MATERIALS AND METHODS

Sub-cloning ET-traps into pIT2 phagemid vector

ET-traps constructs were cloned and displayed in a monovalent phage display system using the 3 + 3 display approach (Figure 1). Each codon-optimized construct (Genscript) was amplified by polymerase chain reaction from its parent plasmid pUC18 using forward and reverse primers with overhangs harboring NcoI and NotI restriction sites. Reaction mixtures were subjected to agarose gel electrophoresis, amplicons were purified with QIAEX II gel extraction kit (Qiagen), and subsequently digested with NcoI/NotI restriction enzymes alongside pIT2 phagemid vector.

Digested inserts were ligated into pIT2 phagemid vector and chemically competent *Escherichia coli* TG1 were transformed with the resulting recombinant phagemids with the heat-shock method. Phages were amplified and rescued by superinfection with KM13 helper phage. PEG/NaCl was used to precipitate and isolate phage clones which were spectrophotometrically quantified with NanoDrop 1000. Phage titers were calculated using equation 1 (derived by Day and Wiseman)[30] and subjected to phage enzyme-linked immunosorbent assay (ELISA).

Verifying ET-traps construct display

MaxiSorp microtiter plate (Nunc) wells were coated with anti-cMyc antibodies (1 μg/mL in PBS) overnight at 4 °C. Wells were blocked with 5% skimmed milk and 100 μL of 5×10^{10} phage clone virions in 0.5% milk/0.1% PBST were added and incubated for 1 h with gentle agitation. After extensive washing, bound phages were detected with anti-M13 monoclonal antibodies conjugated with horseradish peroxidase (GE Healthcare) and chromogenic substrate (3,3′,5,5′- tetramethylbenzidine). Reaction was terminated with 2 M H_2SO_4 and absorbance was measured at 450 nm. The signals generated were later used for normalization of ET-1 binding activity.

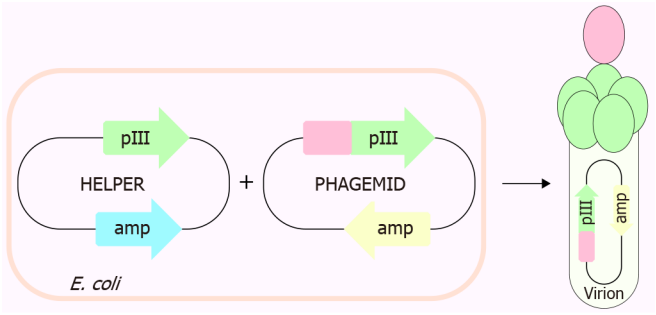
Analysis of ET- traps: ET-1 binding

To increase the adsorption surface area, and thus the detection signal, N-biotin-ET-1 (Phoenix pharma) was coupled to paramagnetic streptavidin beads (MyOne Streptavidin T1, Thermo Fisher Scientific; 10 mg/mL beads) as an alternative to the conventional phage ELISA on 96-well plates. 5 μL of beads were incubated with 2.5×10^{10} ET-traps-displaying phages for 1 h in 500 μL of 0.5% milk/0.1% PBST. After washing and detection reaction, paramagnetic beads were captured on a magnet, and the supernatants were transferred to a 96-well plate for absorbance A_{450} measurements. In parallel, binding of ET-trap constructs to streptavidin beads in absence of ET-1 was analyzed. Absorbance signals were blank-subtracted and normalized according to the relative display levels as determined in anti-cMyc phage ELISA assay.

Creating an Fc-fusion construct and measuring binding affinity to ET-1

The gene for the ET-traps was designed and optimized for expression in mammalian cells (HEK293) prior to being synthesised. The sequence was then sub-cloned into a cloning and expression vector for human Fc fusion proteins.

In brief, HEK293 cells were passaged to the optimum stage for transient transfection. Cells were transiently transfected with the appropriate expression vector and cultured for a further 6-14 d. An appropriate volume of cells was transfected with the aim of obtaining 1-5 mg of purified Fc fusion protein. Cultures were harvested and one-step purification performed using affinity chromatography. For this, culture supernatant containing Fc fusion protein was loaded onto a MabSelect SuRe Protein A



DOI: 10.4239/wjd.v13.i6.434

Figure 1 Depiction of phage display 3 + 3 system.

column at 4 mL/min and washed with PBS pH 7.2. A step elution was performed with sodium citrate buffer (pH 3.0). Eluted protein was neutralised with 10% (v/v) Tris buffer (pH 9.0). Upon purification, the Fc fusion protein was buffer exchanged into PBS pH 7.4. The protein was analysed for purity by SDS-PAGE and concentration determined by UV spectroscopy (at 280 nm).

For the binding affinity measurement, we employed the use of the Octet Red96 system (Patel *et al* 2013). In brief, the kinetics of the selected ET-traps binding to biotinylated ET-1 (Phoenix Pharmaceuticals) was determined using the Octet Red96 system (ForteBio, Menlo Park, CA). The buffer for the assays was PBS with 0.01% (w/v) bovine serum albumin and 0.002% Tween20. The measurements were carried out at 30 °C. 1 μg/mL bio-ET-1 was captured on dip-and-read streptavidin sensors, followed by binding of the selected ET-traps at 500 nM concentration. The ForteBio Octet analysis software (ForteBio, Menlo Park, CA) was used to generate the sensorgram.

RESULTS

Previous work identified a strong binder to ET-1[31]. In this study, we tested different sequence combinations of ET-traps that could also bind ET-1. We performed phage display experiments to ascertain this.

Phage display experiments

Phage display is a powerful technique commonly used today to identify different protein–protein interactions. We displayed individual ET-traps in a monovalent setting (*i.e.* 3 + 3 display type[32]) to prevent avidity effects on binding to ET-1. The cMyc-tag peptide present in the linker region that tethers ET-traps to the anchoring phage coat protein p3 allows for assessment of constructs' display levels by phage ELISA against anti-cMyc antibody. These were, in turn, used to normalize signals from phage ELISA where binding of ET-traps to biotinylated ET-1 was analyzed (Figure 2).

The construct ζ gave strong binding to ET-1 in phage display experiments, but the Fc-fusion molecule was not stable and the results could not be replicated with the soluble fusion protein. The phage experiments confirmed that construct β indeed gave consistent, high binding. It was further observed that in the form of an Fc-fusion construct β showed high binding affinity to ET-1 consistently (Figure 3).

DISCUSSION

This paper discusses the characterization of ET-traps that might be useful in the treatment of ET-1 related diseases or disorders, such as preeclampsia, cardiovascular diseases, chronic kidney disease, diabetes or neurodegenerative disorders[11,18,19,33-36]. Previous work has shown that the use of the selected ET-traps gave a therapeutic effect on reducing different markers of diabetes-induced disease pathology[12,13]. The ET-traps helped reduce different markers of diabetes disease pathology, such as over-expression of ECM proteins, proteinuria and tissue damage to kidneys and heart. This effect was found to be statistically significant both *in vitro* and *in vivo*. The ET-traps were designed based on a previous study[22]. The purpose of this study was to ascertain our selection of the ET-traps. Both ECL2 and ECL3, including the flanking transmembrane regions, were found to play an important role in ligand selection[22]. Further, residues in the intracellular loop and of the C-terminus are important for ET-1 binding[22]. These domains were used to create the final ET-traps that gave an efficacious, therapeutic effect in our proof-of-concept studies done both at the *in vitro* and *in vivo* levels in the diabetes disease space[12,13]. The final design of our ET-traps ensured that the selected ET-traps do not bind the ET-1 paralogs, which is important for selective activity and thus fewer potential adverse effects.

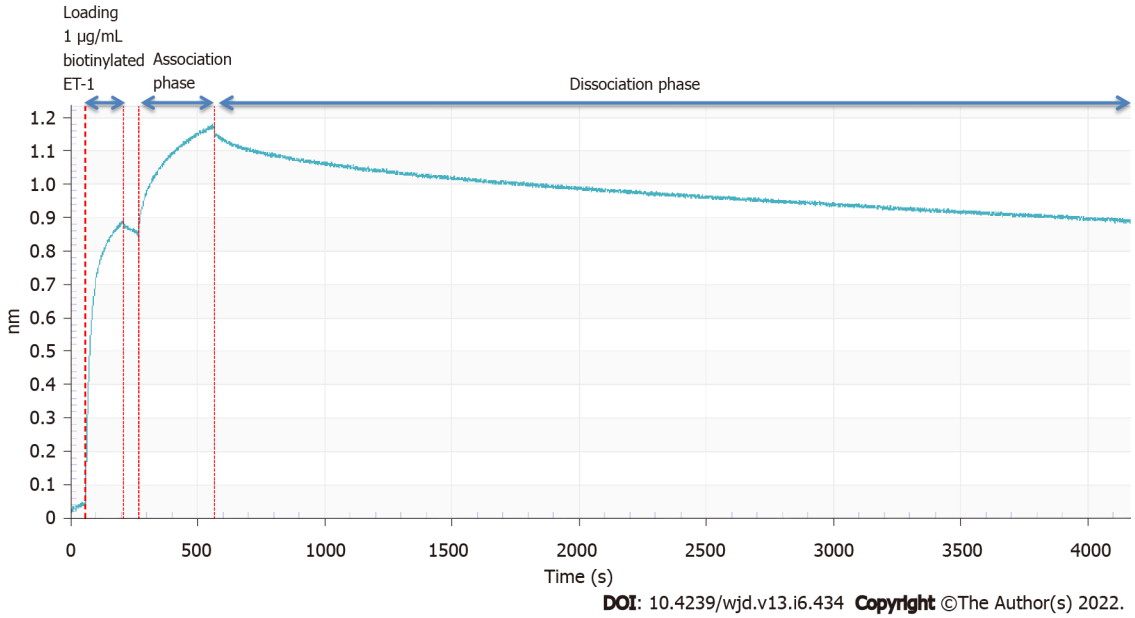


Figure 2 Sensorgram of Fc-β binding to biotinylated endothelin-1. Representative plot shows the binding assay revealed that our selected endothelin (ET)-traps bind strongly to ET-1, displaying double-digit picomolar binding affinity (an average of 73.8 picoMolar, *n* = 6).

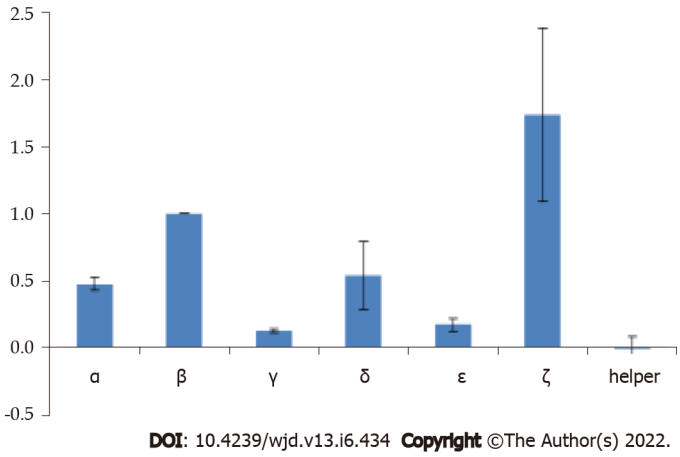


Figure 3 Average relative endothelin-1 binding of endothelin-traps constructs expressing clones using phage display. The final signals are blank-subtracted and normalized to individual expression levels.

We began with phage display experiments to test different combinations of potential ET-traps, including the previously selected ET-traps that were used to perform the proof of concept (PoC) work [12,13]. These experiments confirmed the selection of the most efficient ET-traps. Phage experiments were performed on different combinations of sequences (Figure 3). These experiments allowed us to confirm our final ET-traps selection and we then proceeded to test the binding of its cognate Fc fusion to ET-1.

As ET-1 is abundant in the body while ET-2 is almost undetectable, ET-1 was more convenient to research; this assumption has meant ET-2 is relatively under-researched[26]. However, recent research evidence suggests distinct roles and features of ET-2. In mice with the ET-2 gene knocked-out, the animals displayed growth retardation, and were hypothermic and hypoglycemic, which resulted in early mortality[37].

ET receptor antagonists (ERAs) can have a deleterious effect on physiological ET-2 functions by completely blocking the receptors and thereby inhibiting the physiological actions of ET-2. With our ET-traps, we would overcome this; the ET-traps specifically bind to ET-1 and do not block the receptors to effect ET-2 actions like an inhibitor to the ET system might do. The ET-traps have been designed to specifically bind ET-1.

Aberrations in the *EDN3* gene that is responsible for producing ET-3 have been associated with congenital disorders involving neural crest-derived cells, like Hirschsprung disease and Waardenburg syndrome[38,39]. This shows that ET-3 is one of the important peptides of the ET family, which is

involved in various developmental processes. Further, use of ERAs would essentially block the function of this molecule thereby potentially causing serious birth defects. This again precludes completely blocking the physiological functions of ET-3, as it is one of the important factors for essential developmental processes. Again, with the ET-traps, we would not completely be blocking the ET system, rather just targeting elevated ET-1 Levels upstream. As found in this study, the selected ET-traps bind ET-1 with a high binding affinity in the double-digit picomolar range (an average of 73.8 rM, n = 6). This was also previously found and reported by Jain *et al*[12] in their diabetes PoC study. This work showed that the selected ET-traps have an efficacious, therapeutic effect in ameliorating diabetes disease pathology [12,13]. This was not associated with any toxic effects as evinced by the toxicology data. This corroborates that the selected ET-traps are efficacious at the working concentration and specific to just ET-1.

CONCLUSION

The ET-traps were designed to specifically bind ET-1. The results of this study confirm that our selected ET-traps specifically bind to ET-1. This is in agreement with previous PoC studies that detected no toxic effects of the selected ET-traps at the working concentration. This is an important factor for the potential use of ET-traps as a therapeutic.

ARTICLE HIGHLIGHTS

Research background

Endothelin (ET)-1 is a very potent vasoactive peptide that is significantly elevated in different diseases.

Research motivation

We wanted to develop a cure that would target this peptide and would help save millions of lives around the world.

Research objectives

To develop a tool that specifically targets ET-1.

Research methods

We employed phage display and binding assays.

Research results

A very high binding affinity was observed for our selected tool.

Research conclusions

Developed a potent tool targeting ET-1.

Research perspectives

A new target in drug discovery and development.

ACKNOWLEDGEMENTS

This paper is dedicated to Dr. M.L. Mehrotra. We would also like to thank Mr. Ashok Jain for all his dedication and support during this difficult time.

FOOTNOTES

Author contributions: Jain A and Bozovičar K contributed equally to this work; Jain A, Bozovičar K, Mehrotra V, Bratkovič T performed the experimental analyses and contributed towards writing the article and Johnson M and Jha I revised it critically for important intellectual content.

Institutional review board statement: This study was approved by ET-traps Limited.

Conflict-of-interest statement: The authors have no conflict of interest.

Data sharing statement: Data available from corresponding author, Dr. Arjun Jain at arjun@et-traps.co.uk.

Country/Territory of origin: United Kingdom

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 June 15; 13(6): 442-453

DOI: 10.4239/wjd.v13.i6.442

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Preparation and hypoglycemic effects of chromium- and zinc-rich *Acetobacter aceti*

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ajjan RA, United Kingdom; Lee CC, Taiwan; Yun JH, South Korea

A-Editor: Liu X, China

Received: March 1, 2022
Peer-review started: March 1, 2022
First decision: April 17, 2022
Revised: April 25, 2022
Accepted: May 21, 2022
Article in press: May 21, 2022
Published online: June 15, 2022

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Abstract

BACKGROUND

At present, there is no ideal method to cure diabetes, and there are few reports on the treatment of diabetes with probiotics.

AIM

To propose a method for preparing a new type of chromium- and zinc-rich *Acetobacter aceti* (*A. aceti*) and explore its ability to enhance the hypoglycemic effects of probiotics in the treatment of diabetes.

METHODS

A. aceti was cultured in a liquid medium that contained chromium trichloride and zinc chloride, both at a concentration of 64 mg/mL, with the initial concentration of the bacterial solution 1 × 10^4 CFU/mL. After the bacterial solution had been inducted for 48 h, the culture media was changed and the induction was repeated once. The levels of chromium and zinc in the bacteria were detected by inductively coupled plasma mass spectrometry, and the contents of NADH and glucose dehydrogenase were determined using an NAD/NADH kit and glucose dehydrogenase kit, respectively. Streptozotocin was used to establish a mouse model to evaluate the hypoglycemic effects of the proposed chromium- and zinc-rich *A. aceti*. Ten-times the therapeutic dose was administered to evaluate its biological safety. The effect on MIN6 islet cells was also assessed *in vitro*.

RESULTS

The levels of chromium metal, metallic zinc, NADH coenzyme, and glucose dehydrogenase in *A. aceti* prepared by this method were 28.58-34.34 mg/kg, 5.35-

7.52 mg/kg, 5.13-7.26 μM, and 446.812-567.138 U/g, respectively. The use of these bacteria resulted in a better hypoglycemic effect than metformin, promoting the repair of tissues and cells of pancreatic islets *in vivo* and facilitating the growth of MIN6 pancreatic islet cells and increasing insulin secretion *in vitro*. Ten-times the therapeutic dose of treatment was non-toxic to mice.

CONCLUSION

Chromium trichloride and zinc chloride can be employed to induce the preparation of chromium- and zinc-rich *A. aceti*, which can then promote the hypoglycemic effect found in normal *A. aceti*. The bacteria biotransforms the chromium and zinc in a way that could increase their safety as a treatment for diabetes.

Key Words: *Acetobacter aceti*; Chromium; Zinc; Enrichment; Blood sugar decrease

Core Tip: At present, there are no ideal drugs to treat diabetes. Acetobacter and other probiotics can be used in the treatment of diabetes, but their effect is not significant. The focus of this study is to determine if enriching chromium and zinc in *Acetobacter aceti* could enhance the hypoglycemic effect of this probiotic. In this study, metal compounds were used to induce *A. aceti* to enrich chromium and zinc concentrations, and the effects of these metal-enriched bacteria on the hypoglycemic effect were assessed. These chromium- and zinc-rich bacteria were able to increase the hypoglycemic effect and, due to low toxicity, have good prospects as a treatment for diabetes.

Citation: Huang YY, Qin XK, Dai YY, Huang L, Huang GR, Qin YC, Wei X, Huang YQ. Preparation and hypoglycemic effects of chromium- and zinc-rich *Acetobacter aceti*. *World J Diabetes* 2022; 13(6): 442-453
URL: https://www.wjgnet.com/1948-9358/full/v13/i6/442.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i6.442

INTRODUCTION

Diabetes mellitus is a disease of chronic glucose metabolism disorders[1,2]. In 2020, the world's population with diabetes was about 495 million, and this is expected to increase to 700 million by 2045 [3,4]. The pain and burden of diabetes and its complications are major health and socio-economic concerns for people all over the world. Diabetic patients are often challenged by serious complications, such as diabetic nephropathy, diabetic retinopathy, cardiovascular system disease, *etc*, which pose a serious threat to human life and health[5-7]. Patients with type 2 diabetes mellitus (T2DM) account for 90% to 95% of all diabetic patients, making T2DM an important focus of much research into the epidemiology of diabetes. Scholars have heavily researched the causes of diabetes and methods for its prevention, for many years; however, there remains no effective cure, due to the disease's unclear causes and complicated pathogenesis. Moveover, its underlying mechanism has yet to be fully elucidated[8,9]. The current comprehensive prevention and treatment measures for diabetes mainly include diet control, exercise, and drug therapy[10-12].

The drugs used for the treatment of diabetes are mainly chemical agents, with few biological drugs available. Health drinks, including kombucha (which contains yeast, lactic acid bacteria, and acetic acid bacteria and their metabolites) and black tea, can lower blood sugar, blood pressure, blood lipids, *etc*, and exert certain auxiliary effects on patients with hypertension, hyperlipidemia, and hyperglycemia; the main active ingredients in these drinks that lowers blood sugar are D-glucaric acid-1, 4-lactone and tea polyphenols, respectively[13]. However, the effect of bacterial metabolites from these drinks on reducing blood sugar is not fully elucidated. For example, *Acetobacter aceti* (*A. aceti*) contains dihydronicotinamide-adenine dinucleotide and glucuronic acid dehydrogenase, among other metabolites, which decompose glucose through glycolysis; however, kombucha fails to improve the effect of reducing blood sugar by fully utilizing the effects of these metabolic enzymes. Therefore, *A. aceti* can only be used as a healthy drink for adjuvant therapy. In order to capitalize on the ability of these bacteria to metabolize glucose, increase the contents of dihydronicotinamide-adenine dinucleotide glucuronic acid dehydrogenase, and other metabolic enzymes, and exert their hypoglycemic effects, their full effectiveness must be realized *in vivo* before these bacteria can be used as a hypoglycemic drug.

To improve the effect of *A. aceti* on lowering blood sugar, the present study provides a method for preparing and applying chromium- and zinc-rich *A. aceti*. This method demonstrated a significant increase in the amount of dihydronicotinamide-adenine dinucleotide, glucuronic acid dehydrogenase, chromium, and zinc inside the cells and other microelements in the specimens we prepared. These

enriched bacteria had a notable repair effect on pancreatic islet cells, promoted insulin secretion, and demonstrated a good hypoglycemic effect. Therefore, chromium- and zinc-rich *A. aceti* can be used as a candidate drug for the treatment of T2DM.

MATERIALS AND METHODS

Materials

Glucose (Guangdong Guanghua Technology Co., Ltd, batch number: 20200403); yeast extract (Beijing Aoboxing Bio-tech Co., Ltd, batch number: 20200422); calcium carbonate (Shanghai Titan Scientific Co., Ltd, batch number: P1260108); agar (Beijing Solarbio Technology Co., Ltd, batch number: 310C022); anhydrous alcohol (Shanghai MacLean Biochemical Technology Co., Ltd, batch number: C11974944); chromium chloride (Shanghai MacLean Biochemical Technology Co., Ltd, batch number: C10717130); zinc chloride (Shanghai MacLean Biochemical Technology Co., Ltd, batch number: C10730413); 50-mL centrifuge tubes and EP tubes (Jiangsu Lexinkang Medical Equipment Co., Ltd); streptozotocin (STZ) (Shanghai McLean Biochemical Technology Co., Ltd, batch number: C20PA038100B); citric acid (Shanghai McLean Biochemical Technology Co., Ltd, batch number: C10723907); sodium citrate (Shanghai McLean Biochemical Technology Co., Ltd, batch number: C10712912); universal pH indicator paper (Hangzhou Test Three Technology Co., Ltd); metformin hydrochloride tablets (Beijing Jingfeng Pharmaceutical Group Co., Ltd, batch number 2004032); glucose test strips (ACCU-CHEK; Roche Diabetes Care GmbH, batch number: 26020933, *etc*); specific pathogen-free (SPF) C57BL/6 mice, aged 6-8 wk (Changsha Tianqin Biological Co., Ltd); *A. aceti*, number: GIM1.67 (Guangdong Microorganism Conservation Centre); and MIN6 cells (China Centre for Type Culture Collection) were used. The Animal Experiment Ethics Approval Number was 20200620.

Preparation of chromium- and zinc-rich A. aceti

A. aceti were revived and cultured in a liquid enriched medium, with the concentration of the bacterial solution OD_{600} = 0.9 to 1.5, which is about 3×10^8 to 5×10^8 CFU/mL. To enrich the bacteria with chromium and zinc, *A. aceti* were cultured in a liquid medium (the concentrations of chromium trichloride and zinc chloride were both 64 mg/mL) with the initial concentration of the bacterial solution set to 1×10^4 CFU/mL and shaken for 48 h at 250 rpm; this cultivation was repeated once. Following the enrichment cultivations, the bacterial solution was collected, centrifuged to remove the supernatant, and washed by phosphate buffer saline (PBS); the precipitate was the chromium- and zinc-rich *A. aceti*.

Detection of coenzymes and metals in A. aceti

Collection of *A. aceti*: Cell suspensions (100 mL) of *A. aceti* were removed and distributed across a sterile 96-microwell plate, with the culture solution used as a blank control. The absorption of the suspension and the *A. aceti* culture solution were measured at 600 nm using the microplate reader and calculated as OD1 and OD2, respectively, with the final OD value of the *A. aceti* suspension taken as the difference OD1-OD2. The *A. aceti* suspension was centrifuged at 8000 rpm for 10 min to remove the supernatant; the precipitate (the *A. aceti*) was then washed once with 1 mL sterile PBS and centrifuged at 13000 rpm for 1 min to remove the supernatant. Thereafter, the precipitate was weighed and resuspended with sterile water for a final concentration of *A. aceti* at 0.1 mg/mL.

Detection of chromium and zinc in *A. aceti*: Collected samples were sent to Shanghai WEIPU Chemical Technology Service Co., Ltd, and the contents of chromium and zinc were detected by inductively coupled plasma mass spectrometry.

Detection of NAD+/NADH in *A. aceti*: The concentrations of NAD+/NADH in the *A. aceti* was determined using the NAD+/NADH assay kit with WST-8 (Beyotime Biotechnology).

Detection of glucose dehydrogenase in *A. aceti*: Solarbio's "glucose dehydrogenase microplate assay kit" was used to detect glucose dehydrogenase concentrations in *A. aceti*, according to the instruction manual.

Construction of a diabetes mouse model: For the preparation of citrate buffer, 2.10 g of citric acid was treated with 100 mL of double distilled water to make a citric acid mother liquor (solution A), after which 2.94 g of trisodium citrate was treated with 100 mL of double-distilled water to make a sodium citrate mother liquor (solution B). Solution A and solution B were mixed in a ratio of 1:1.32 (or 1:1), and the pH value was measured with a pH meter and adjusted from 4.2 to 4.5. This represented the 0.1 mol/L sodium citrate-hydrochloric acid buffer solution required to prepare streptozotocin (STZ).

Seventy SPF grade C57BL/6J female mice, aged 6 wk and weighing 20 ± 2 g, were allowed to eat and drink without restrictions during 5 d of adjustable feeding. Six mice were randomly selected as the normal control group, and the rest were used for model construction.

To prepare the STZ required to inject the mice to cause diabetes, 24 mL of 0.1 mol/L sodium citrate buffer was treated with 120 mg of STZ away from the light (equivalent to a concentration of 5 mg/mL) and placed in an ice environment. The mice were made to fast for 10 h and then treated with STZ at a dose of 0.15 mL/10 g of mouse weight (equivalent to 75 mg/kg). STZ was administered by intraperitoneal injection for 3 consecutive days. Before each intraperitoneal injection, the STZ liquid was carefully pipetted with a 1 mL syringe to mix the precipitate before it was extracted to maintain the STZ concentration. After each intraperitoneal injection, mice were deprived of food and water for 90 min. On the 7th d after the last administration (the mice were made to fast for 10 h before blood collection), blood was collected from their caudal veins, and the fasting blood glucose (FBG) levels of the mice were measured with a Roche glucometer. Pathological models of mice with diabetes were confirmed as having been successfully established when FBG ≥ 16.7 mmol/L.

The diabetic mice were designated from high to low, according to their blood glucose levels, and the model mice were divided into a model group (PBS), positive control group (metformin), metal chromium plus zinc group (concentrations of chromium and zinc calculated as 1×10^{-7} mg/mL and 2×10^{-8} mg/mL, respectively, according to the highest content of chromium- and zinc-rich *A. aceti*), *A. aceti* group (OD = 1), and chromium- and zinc-rich *A. aceti* group (OD = 1). There were six mice in each group, and each was given 0.5 mL of treatment by gavage.

Evaluation of hypoglycemic activity *in vivo*: After the diabetic models were successfully established, the mice were given intragastric administration of treatment on the second day, once a day, for 15 consecutive days. The normal control group and the model group were given the same amount of PBS-water. The positive drug control group was given diformin tablets (ground into powder, prepared into a suspension with reverse osmosis water, and administered intragastrically at 0.320 g/kg/d). The metal chromium plus zinc group was given chromium trichloride (1×10^{-7} mg/mL) and zinc chloride (2×10^{-8} mg/mL) intragastrically. Through general observation, the activity and spirit of the mice, their eating and drinking, urine and feces, and the dryness and wetness of the bedding were observed every day during the 15 d of administration. Starting at the beginning of treatment, fasting blood glucose was measured every 3 d. After 15 d of administration, the mice were fasted for 10 h and blood was collected from the eyeballs. Thereafter, the mice were sacrificed, with the tissue taken from their pancreas islets, fixed with formaldehyde, and processed for hematoxylin and eosin (HE) staining and immunohistochemistry with the apoptosis-related Bax proteins. Tissue from pancreatic islets was also prepared for electron microscopy to analyze the ultrastructural damage and possible repair on tissues and cells of pancreatic islets. The weights of mice were recorded every 3 d.

Evaluation of MIN6 cell growth: *A. aceti* was collected (OD = 10), sonicated, and stored at -20 °C for later use. MIN6 cells, a pancreatic islet cell line, were revived, the cell concentration adjusted to 1×10^5 CFU/mL, and then cultured in 5 mmol/L and 25 mmol/L high-glucose 1640 medium on a 96-well plate with 90 mL/well. After 12 h, the cells were diluted 10-fold and dosed with 10 mL of collected *A. aceti* (with or without metal enrichment; OD = 10), the dose of which was equivalent to 1×10^7 CFU/mL of bacteria. A positive drug control group (diformin tablets) and negative drug control group (PBS) were included. Approximately 24 h after dosing, cell growth was detected with the CCK-8 kit (Beyotime Biotechnology).

Determination of insulin secretion in MIN6 cells: *A. aceti* were collected (OD = 10), sonicated, and stored at -20 °C for later use. MIN6 pancreatic islet cells were revived, the cell concentration adjusted to 1×10^5 CFU/mL, and cultured in 5 mmol/L and 25 mmol/L high-glucose 1640 medium on a 6-well plate with 1.98 mL/well. After 12 h, the cells were dosed with 20 mL *A. aceti* (with or without metal enrichment; OD = 10), the dose of which was equivalent to 1×10^7 CFU/mL of bacteria. A positive drug control group (diformin tablets) and negative drug control group (PBS) were included. Some 24 h after dosing, the cell supernatant was collected, with the insulin content detected by a commercially-available enzyme-immunized mouse insulin ELISA kit.

Detection of glucose processing by A. aceti

Chromium- and zinc-rich *A. aceti* and control *A. aceti* were collected, with the initial concentration adjusted to 1×10^4 CFU/mL. The glucose concentration of the liquid medium was detected with a blood glucometer in *A. aceti* cultured at 30 °C for 12 h, 24 h, 36 h, and 48 h.

Safety evaluation of chromium- and zinc-rich A. aceti

Chromium- and zinc-rich *A. aceti* were collected and adjusted to 10 times the therapeutic dose (OD = 10). Aliquots of 1-mL were administered, respectively, to the mice three times a day for 7 consecutive days, with the body weights and the pathological changes of the organs detected.

RESULTS

Determination of coenzymes and metals in chromium- and zinc-rich A. aceti

The concentration of chromium metal in the *A. aceti* prepared as described in the methods section above was 28.58-34.34 mg/kg, and the zinc metal concentration was 5.35-7.52 mg/kg, both of which were significantly higher than those in the untreated *A. aceti* (chromium: 1.05-2.29 mg/kg; zinc: 0.18-0.26 mg/kg) (Figure 1A). The concentration of NADH was 5.13 to 7.26 mM, which was significantly higher than that in the non-cultured *A. aceti* (0.86 to 1.02 mM) (Figure 1B). The concentration of glucose dehydrogenase was 446.812-567.138 U/g, which was significantly higher than that of non-cultivated *A. aceti* (54.126-93.651 U/g) (Figure 1C).

Evaluation of the therapeutic effects of chromium- and zinc-rich A. aceti on mice with diabetes

After 7 d of treatment, the diabetic mice (mice with FBG ≥ 16.7 mmol/L) treated with chromium- and zinc-rich *A. aceti* were found to be in a good mental state, with bright eyes and normal activity, normal intake of food and water, urinate, and normal defecation, with their beddings dry. The fasting blood glucose was detected every 3 d after the initial administration of treatment, and the diabetic mice treated with chromium- and zinc-rich *A. aceti* (OD = 1) had significantly lower blood glucose than mice in both the positive drug control group (diformin tablets) and the metal chromium plus zinc group (Figure 2).

After 15 d of treatment, HE staining of the pancreas tissue indicated fewer cells undergoing apoptosis, less structural atrophy, and hardly any vacuoles detected in the chromium- and zinc-rich *A. aceti* group (OD = 1) (Figure 3). Immunohistochemistry analysis of apoptosis-related Bax proteins confirmed that the apoptosis of islet cells was significantly reduced (Figure 3). Electron microscopy of pancreas islet tissue confirmed that the ultrastructural damage was alleviated after treatment with the chromium- and zinc-rich *A. aceti,* with a small expansion range of endoplasmic reticulum, slightly swollen mitochondria, and the amount of autophagic vacuolization significantly reduced (Figure 3).

The body weights of the mice after treatment with chromium- and zinc-rich *A. aceti* were recorded every 3 d. The weights of diabetic mice in the chromium- and zinc-rich *A. aceti* (OD = 1) were found to recover well, with no significant difference from those of the positive drug control group (metformin), as displayed in Figure 4.

Evaluation of chromium- and zinc-rich A. aceti on pancreatic islet cells MIN6

Chromium- and zinc-rich *A. aceti* promoted the growth of MIN6 cells significantly better than both the positive and negative control groups in the high glucose 1640 medium, as indicated by cytotoxicity measured with the CCK-8 kit (Figure 5). This result implied that chromium- and zinc-rich *A. aceti* could promote the growth of MIN6 pancreatic islet cells.

The insulin content of the supernatant in MIN6 cells incubated in the 25 mmol/L high-glucose 1640 medium treated with chromium- and zinc-rich *A. aceti* (OD = 10) group was significantly higher than that in the positive and negative control groups (Figure 6). This result implies that chromium- and zinc-rich *A. aceti* could promote insulin secretion of MIN6 islet cells.

Detection of the capacity of chromium- and zinc-rich A. aceti for processing glucose

The glucose content in medium containing chromium- and zinc-rich *A. aceti* and control *A. aceti* both decreased significantly as incubation times increased; however, this decrease was significantly more pronounced for the chromium- and zinc-rich *A. aceti* compared to the *A. aceti* group (Figure 7). This result implies that chromium- and zinc-rich *A. aceti* are better at decomposing glucose than the untreated bacteria.

Safety evaluation of chromium- and zinc-rich A. aceti

Ten times the dose of chromium- and zinc-rich *A. aceti* was administered to mice by gavage with no change detected in the weights and no pathological damage found in the liver, spleen, kidneys, and stomach (Figure 8). This result implies that *A. aceti* is biosafe.

DISCUSSION

At present, the incidence of diabetes is high, with no known cure. The conventional therapy is long-term use of hypoglycemic drugs and symptomatic treatment. However, in the long-term treatment process, drugs are prone to resistance among non-compliant patients who fail to adhere to treatment regimens. Therefore, in addition to helping patients control blood sugar long-term, it is vital that we clearly identify the risk factors for diabetes to prevent patients from developing this disease. An important area that may be significant in both the long-term treatment and prevention of diabetes is the use of supplemental micronutrients, as diabetic patients are often deficient in B vitamins and micronutrients, such as chromium, zinc, and selenium[14-16].

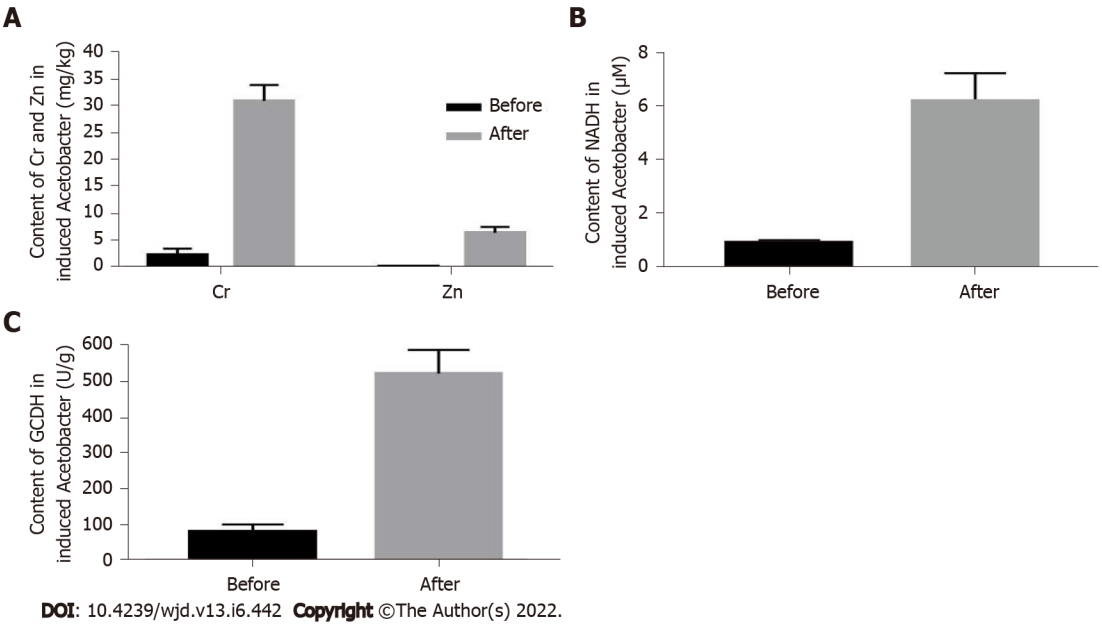


Figure 1 Detection of hypoglycemic components of chromium- and zinc-rich *Acetobacter aceti*. A: Concentration of chromium and zinc; B: Concentration of dihydronicotinamide-adenine dinucleotide; C: Concentration of glucuronic acid dehydrogenase.

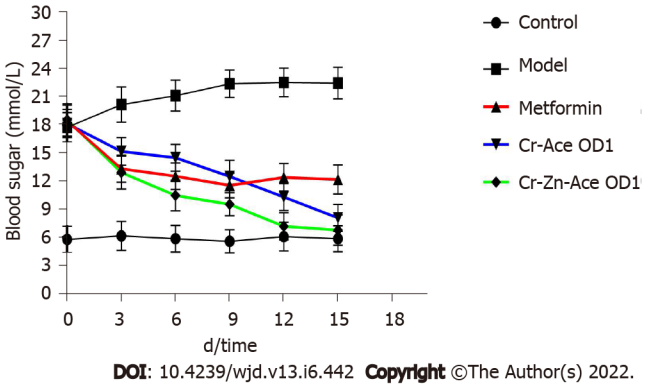
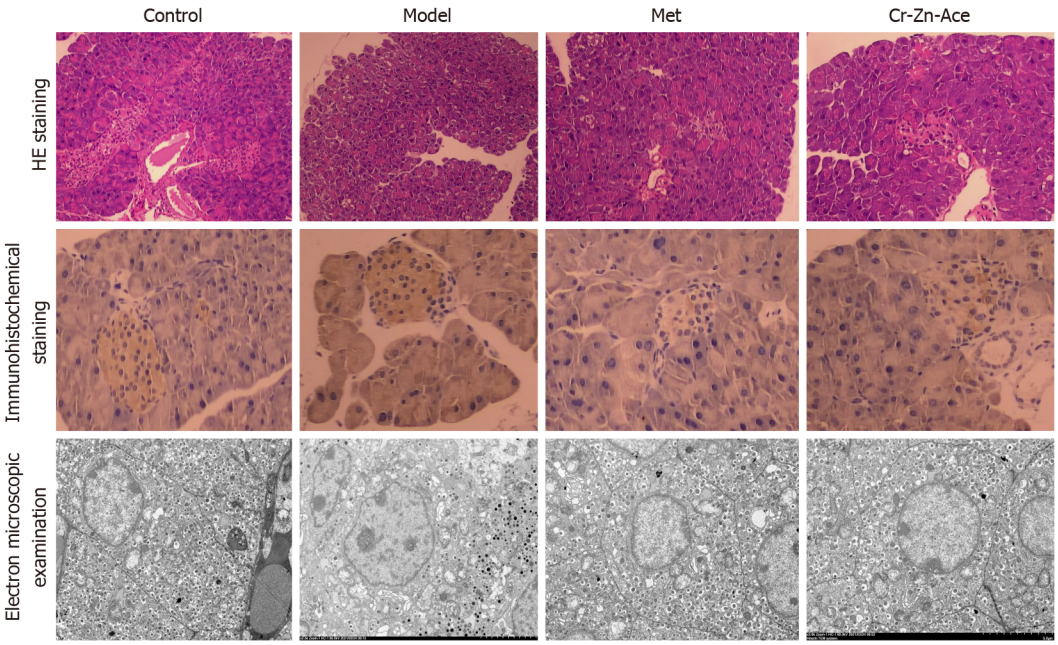


Figure 2 Evaluation of hypoglycemic effects of chromium- and zinc-rich *Acetobacter aceti* on mice with diabetes.

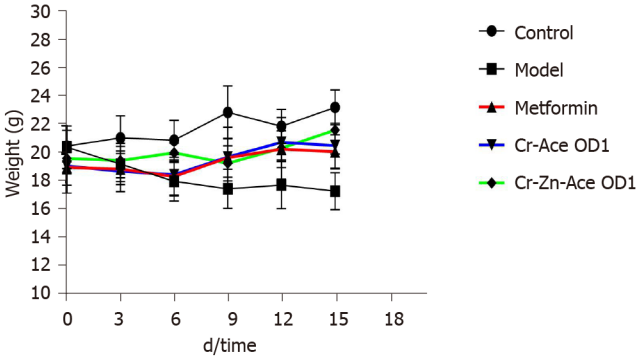
Chromium was designated as an essential microelement for the human body in 1989. The body absorbs Cr^{3+} mainly from food, including meat, whole grain, millet, pepper, *etc*. The daily intake for adults recommended by the United States Food and Drug Administration's Center for Food Safety and Applied Nutrition Food Safety Recommendations Committee is 25-35 μg (as of 2001). Studies have found that plasma chromium levels are negatively correlated with the risk of type 2 diabetes and prediabetes. Chromium deficiency may lead to impaired glucose tolerance, insulin resistance, and elevated blood sugar. Chromium levels in the human body will decrease with age, with chromium deficiency becoming severe in old people[17-19]. According to evidence-based medicine meta-analysis studies, chromium increased insulin sensitivity mainly by activating insulin receptor kinase activity, inhibiting phosphatase activity, and increasing phosphorylation of insulin receptors[20,21]. Low molecular weight chromium binding substance was found to be combined with insulin receptors and activate tyrosine kinase activity on insulin P receptors, thus enhancing insulin signal transduction. However, at an insulin concentration of 100 nmol/L, the tyrosine kinase activity in insulin receptors was significantly enhanced if insulin was dosed with chromium[22,23]. In addition, it was found that chromium could also promote the translocation of GLUT4 to the cell membrane by activating protein kinase B and adenosine monophosphate to activate protein kinase signaling pathways and by activating the activity of p38 mitogen-activated protein kinase[24,25]. Despite much related research, the specific mechanism of lowering blood sugar through chromium supplementation remains unclear.

Zinc ions are an important part of the insulin molecule, maintaining the stability and biological effects of insulin[26-28]. Zinc plays a key role in the synthesis, storage, and secretion of insulin in pancreatic β cells and can increase the activity of the insulin signaling pathway. However, zinc deficiency can lead to a decrease in insulin secretion[29,30]. In addition, the formation of hexamers that contain zinc is



DOI: 10.4239/wjd.v13.i6.442

Figure 3 Repair effect of chromium- and zinc-rich *Acetobacter aceti* on tissues and cells of pancreatic islets.

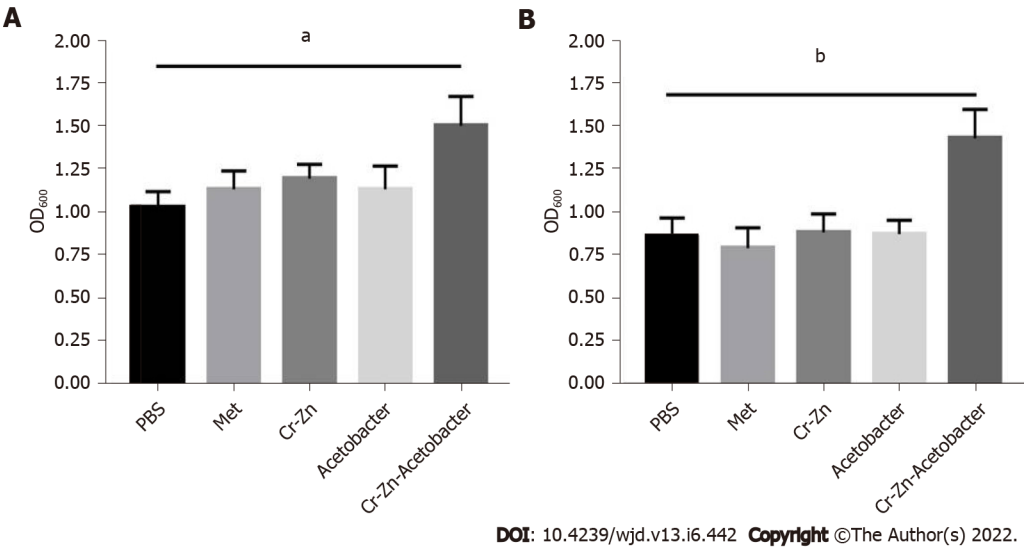


DOI: 10.4239/wjd.v13.i6.442

Figure 4 Effects of chromium- and zinc-rich *Acetobacter aceti* on weight recovery of diabetic mice.

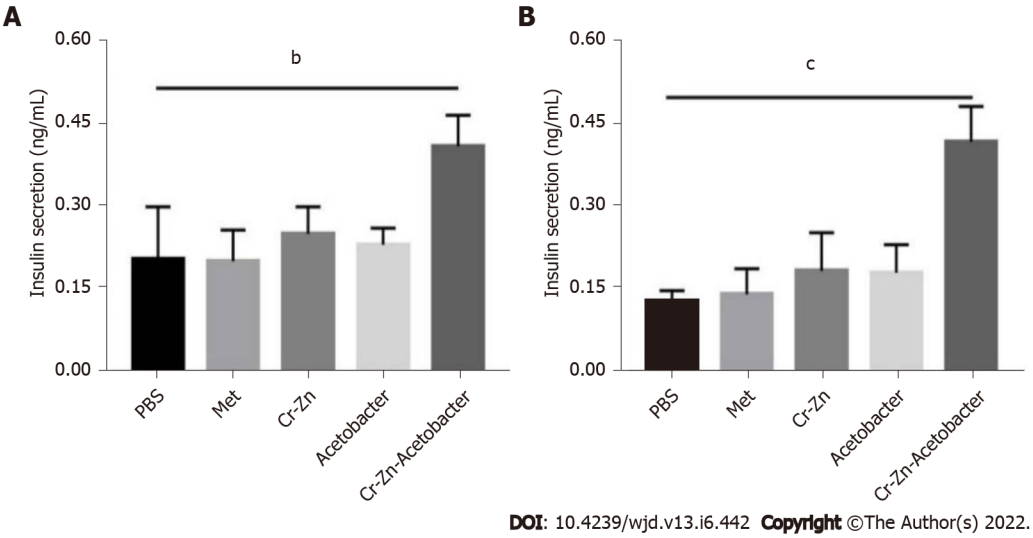
required in the synthesis of insulin; therefore, zinc deficiency will lead to restricted insulin synthesis and reduced insulin sensitivity, thus increasing the risk of diabetes[31,32]. Studies have found that zinc lowers blood sugar mainly through antioxidant responses, inhibition of inflammatory factors, and anti-apoptosis effects[30,33]. Intracellular zinc has also been found to be regulated by zinc transporters, with the uptake, storage, and distribution regulated by metallothioneins[34,35]. Studies have found that zinc can inhibit the inflammatory response and has an anti-apoptosis effect at a concentration higher than 100 μmol/L[36]. Therefore, increasing the intake of zinc can increase the level of metallothionein, which helps mediate anti-apoptosis, *etc*[31,37]. Some scholars have suggested that the effects of a high-zinc intake on lowering blood sugar may be associated with its ability to reduce variation in zinc transporters[38]. Therefore, given the important role of zinc in glycemic control, a deficiency of zinc can lead to glucose metabolism disorders, while high intake may reduce the risk of glucose metabolism disorders and diabetes.

Above all, chromium and zinc supplementation can stabilize blood sugar in an indisputable manner. The key is how to supplement chromium and zinc through a proper, safe, and scientific approach. In the present study, chromium trichloride, zinc chloride, and *A. aceti* were co-cultured to induce the production of chromium- and zinc-rich *A. aceti* following simple protocols with high yield. Chromium- and zinc-rich *A. aceti* prepared using this method not only helped *A. aceti* exert the effect of decomposing glucose but also enhanced the hypoglycemic effect seen in untreated *A. aceti*. Because the chromium and zinc was transformed by the bacteria, biological safety was ensured, which means that it is possible that this can be used as a new hypoglycemic biological drug. The hypoglycemic mechanism of chromium- and zinc-rich *A. aceti* was preliminarily explored, with findings of these bacteria predom-



DOI: 10.4239/wjd.v13.i6.442

Figure 5 Effects of chromium- and zinc-rich *Acetobacter aceti* on promoting growth of pancreatic islet cells MIN6. A: 5 mmol/L glucose; B: 25 mmol/L glucose. $^{a}P < 0.05$; $^{b}P < 0.01$.



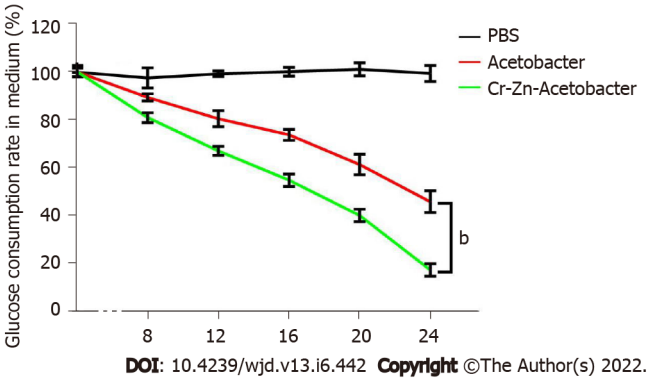
DOI: 10.4239/wjd.v13.i6.442

Figure 6 Effects of chromium- and zinc-rich *Acetobacter aceti* on facilitating growth and insulin secretion of islet MIN6 cells. A: 5 mmol/L glucose; B: 25 mmol/L glucose. $^{b}P < 0.01$; $^{c}P < 0.001$.

inantly leading to increased dihydronicotinamide-adenine dinucleotide and glucuronide dehydrogenase levels in *A. aceti*, enhancing the ability to degrade glucose. In addition, its hypoglycemic mechanism was found to be not much different from those of chromium and zinc, which are metal microelements. However, because the source of nutrition for the growth of *A. aceti* is ethanol, these bacteria do not survive for long in the body, and, therefore, they cannot exert long-term hypoglycemic effects, which will be further addressed in future research.

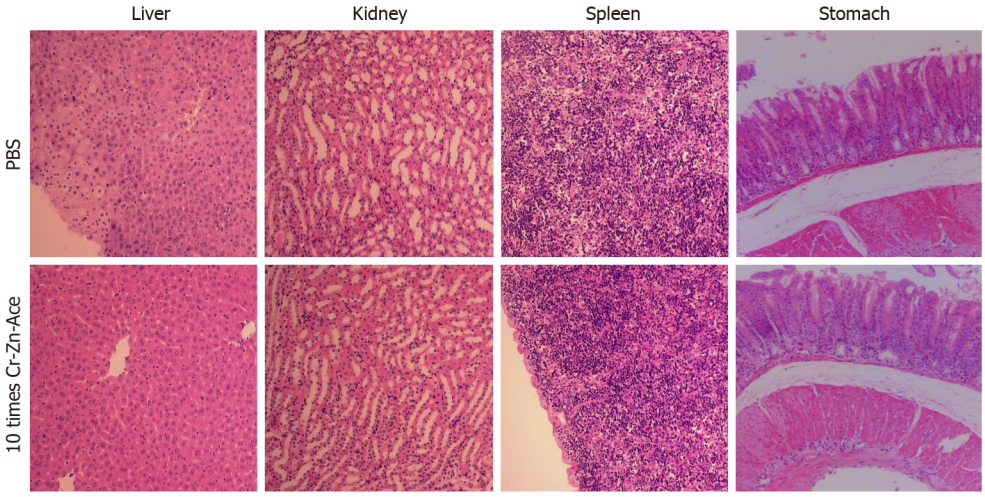
CONCLUSION

Chromium trichloride and zinc chloride can be employed to induce the preparation of chromium- and zinc-rich *A. aceti*, which has a significantly enhanced hypoglycemic effect relative to normal *A. aceti* and can biotransform chromium and zinc in a way that improves the safety of administering these metals as a treatment for diabetes.



DOI: 10.4239/wjd.v13.i6.442

Figure 7 Capacity of chromium- and zinc-rich *Acetobacter aceti* for processing glucose. $^{b}P < 0.01$.



DOI: 10.4239/wjd.v13.i6.442

Figure 8 Safety evaluation of chromium- and zinc-rich *Acetobacter aceti*.

ARTICLE HIGHLIGHTS

Research background

At present, there are no ideal drugs to treat diabetes. Acetobacter and other probiotics can play a role in the treatment of diabetes; however, their effect is not significant. In this study, metal compounds were used to enrich Acetobacter with chromium and zinc in an effort to enhance the hypoglycemic effect of these bacteria.

Research motivation

This research provides a theoretical basis for the application of new chromium- and zinc-rich *Acetobacter aceti* (*A. aceti*) to treat diabetes.

Research objectives

To prepare a new type of chromium- and zinc-rich *A. aceti* and explore its hypoglycemic effects on enhancing the application of probiotics in the treatment of diabetes.

Research methods

A. aceti was cultured in a liquid medium that contained chromium trichloride and zinc chloride.

Research results

A new type of chromium and zinc rich *A. aceti* was successfully prepared.

Research conclusions

Chromium- and zinc-rich *A. aceti* has a significantly enhanced hypoglycemic effect and can biotransform chromium and zinc to improve safety for administering these metals as a treatment.

Research perspectives

The new method described has very good application prospects.

FOOTNOTES

Author contributions: Huang YY was responsible for the experimental research; Qin XK, Dai YY, Huang L, and Huang GR consulted the literature and wrote the first draft, then corrected and improved the manuscript; Wei X and Huang YQ designed, checked, modified, and finalized the manuscript, contributed equally to this work, and agreed to serve as co-corresponding authors; All authors proofread the revised manuscript.

Supported by the Fourth Self-funded Projects of Baise Scientific Research and Technology Development Plan, No. 20203402; and School-level Scientific Research Project of Youjiang Medical University for Nationalities, No. yy2021sk029.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Youjiang Medical University for Nationalities.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: All experimental data are presented in the paper.

ARRIVE guidelines statement: The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

Country/Territory of origin: China

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

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World J Diabetes 2022 June 15; 13(6): 454-465

DOI: 10.4239/wjd.v13.i6.454

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Immediate-release tofacitinib reduces insulin resistance in non-diabetic active rheumatoid arthritis patients: A single-center retrospective study

Chrong-Reen Wang, Hung-Wen Tsai

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: El-Shishtawy MM, Egypt; Shao JQ, China
A-Editor: Yao QG, China

Received: January 14, 2022
Peer-review started: January 14, 2022
First decision: April 18, 2022
Revised: April 18, 2022
Accepted: May 28, 2022
Article in press: May 28, 2022
Published online: June 15, 2022

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Abstract

BACKGROUND

An increased risk of insulin resistance (IR) has been identified in rheumatoid arthritis (RA), a chronic inflammatory disorder with elevated levels of pathogenic cytokines. Biologics targeting proinflammatory cytokines can control the disease and improve insulin sensitivity in RA. Although Janus kinase (JAK) signaling can regulate cytokine receptors and participate in RA pathogenesis, it remains to be elucidated whether there is a reduction of IR in such patients under JAK inhibitor (JAKi) therapy.

AIM

To study the effect of JAKi treatment on the reduction of IR in RA patients with active disease.

METHODS

A retrospective study was carried out from April 1, 2017 to March 31, 2021 in a population of non-diabetic patients with active RA who were undergoing tofacitinib (TOF) therapy with 5 mg twice-daily immediate-release formulation.

RESULTS

Fifty-six RA patients, aged 30 years to 75 years (mean ± SD: 52.3 ± 11.1) with disease activity score 28 values ranging from 4.54 to 7.37 (5.82 ± 0.74), were classified into high-IR (> 2.0) and low-IR (≤ 2.0) groups based on their baseline homeostatic model assessment (HOMA)-IR levels. They had no previous exposure to JAKi, and received TOF therapy for no less than 6 mo. In 30 patients who were naïve to biologics, after a 24-week therapeutic period, the high-IR

group showed reduced HOMA-IR levels (3.331 ± 1.036 *vs* 2.292 ± 0.707, $P < 0.001$). In another 26 patients who were exposed to tumor necrosis factor-α or interleukin-6 blockers, the high-IR group, despite having achieved a decrease but with lower magnitude than in naïve patients, showed reduced HOMA-IR levels (2.924 ± 0.790 *vs* 2.545 ± 1.080, $P = 0.018$).

CONCLUSION

In this retrospective study, reduced IR was achieved in non-diabetic active RA patients following 24 wk of TOF therapy.

Key Words: Insulin resistance; Rheumatoid arthritis; Diabetes mellitus; Tofacitinib; Janus kinase inhibitor

Core Tip: An increased risk of insulin resistance (IR) has been identified in rheumatoid arthritis (RA), a chronic inflammatory disorder with elevated levels of pathogenic cytokines. In addition to controlling RA activity, biologics targeting proinflammatory cytokines have been shown to reduce IR, while it remains to be elucidated whether Janus kinase inhibitor therapy can cause IR reduction in such patients. This retrospective study carried out in non-diabetic active RA patients classified into high-IR and low-IR groups before tofacitinib (TOF) therapy demonstrated reduced IR by 24 wk of TOF treatment in the active RA patients with high baseline IR status.

Citation: Wang CR, Tsai HW. Immediate-release tofacitinib reduces insulin resistance in non-diabetic active rheumatoid arthritis patients: A single-center retrospective study. *World J Diabetes* 2022; 13(6): 454-465
URL: https://www.wjgnet.com/1948-9358/full/v13/i6/454.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i6.454

INTRODUCTION

A critical mechanism causing diabetes development is the resistance of target cells to the action of insulin, with ineffective strength of signaling from the receptor to the final action substrates and requiring beyond-normal insulin concentrations to maintain euglycemic status[1,2]. Insulin resistance (IR) manifests from a blockade of tissues to the insulin action upon the uptake, metabolism or storage of glucose, a common feature of human disorders such as diabetes, hyperlipidemia, metabolic syndrome, fatty liver, and obesity[1]. Furthermore, an increased risk of IR has been identified in various inflammatory disorders with increased levels of proinflammatory cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α[3].

Rheumatoid arthritis (RA), a chronic inflammatory disorder with elevated levels of proinflammatory cytokines, has been demonstrated to be associated with IR during its activity[4]. TNF-α is involved in IR pathogenesis through the phosphorylation of inhibitory serine residue of insulin receptor substrate-1 (IRS-1) and reduction of tyrosine phosphorylation of IRS-1 and the β-subunit of the insulin receptor[5,6]. Inactivation of TNF-α by use of recombinant soluble receptor fusion proteins or monoclonal antibodies for IR reduction has been successfully demonstrated in active RA[7]. IL-6 can exert a negative influence on insulin signaling by decreasing tyrosine phosphorylation of IRS-1, inducing recruitment of IRS-1 to its receptor complex for serine phosphorylation, and reducing autophosphorylation of tyrosine residues in the insulin receptor[8,9]. Under treatment with tocilizumab (TCZ; an IL-6 receptor antibody) to inhibit IL-6 signaling in RA, decreased IR was identified in an investigation of 221 active patients as well as in other studies with smaller sample sizes[10-13]. IL-1β is able to impair insulin signaling through activation of the IKKβ/NF-κB pathway to target IRS-1 through serine phosphorylation[14,15]. Anakinra, an IL-1 receptor antagonist, has been shown to reduce IR in active RA with comorbid type 2 diabetes[16,17]. Altogether, these observations indicate that, biologic therapy targeting pathogenic cytokines can not only control disease activity but also improve insulin sensitivity in active RA patients.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, including JAKs 1 to 3, STATs 1 to 6, and tyrosine kinase 2, regulates many cytokine and hormone receptors with pathogenic roles in a variety of inflammatory disorders[18]. Notably, different cytokine receptors can recruit their own combinations of JAKs and STATs to activate distinct processes in individual targeted cells, while antagonizing a JAK can suppress more than one cytokine pathway, expanding the efficacy in using such an antagonist in cytokine-targeted therapy[19]. Notably, tofacitinib (TOF) is the first small-molecule pan-JAK inhibitor (JAKi) targeting JAKs 1 to 3[20]. It has been approved by the United States' Federal Food and Drug Administration in 2012 and by European Medicines Agency in 2017 for the treatment of RA patients with moderate to high activity and an inadequate response to methotrexate

[21]. This JAKi can act on the JAK/STAT pathway to block the intracellular signaling of multiple cytokines and hormones involved in the pathogeneses of RA and IR[20,22]. In RA patients, significantly reduced circulating levels of pro-inflammatory cytokines IL-6 and TNF-α, two crucial mediators of IR, were observed since week 4 after initiation of TOF therapy[23,24]. Furthermore, in a recent large-scale survey of 10019 RA patients with type 1 or 2 diabetic co-morbidity, the diabetic treatment intensification, *i.e.* addition of a new anti-diabetic medication, was found to be lower for those using TOF than for those using other TNF-α inhibitors or non-TNF-α-targeted biologics[25]. Based on the above findings, there is a therapeutic potential to reduce the IR in active RA patients by TOF therapy.

In this retrospective investigation, the effect of TOF treatment (specifically, 5 mg twice-daily immediate-release formulation) on IR reduction was investigated in 56 non-diabetic patients with active RA, naïve or exposed to biologic therapy and classified into high- and low-IR groups according to the baseline levels of the homeostatic model assessment (HOMA)-IR score.

MATERIALS AND METHODS

Study design and patients

This study was carried out to analyze the effect of TOF on IR in active RA patients who met the 2010 American College of Rheumatology/European League Against Rheumatism (EULAR) classification criteria[26]. Each patient received regular monthly follow-up at an outpatient rheumatology clinic of National Cheng Kung University Hospital from April 1, 2017 to March 31, 2021. This study was approved by the Institutional Review Board and conducted according to the guidelines of Declaration of Helsinki. Before receiving the 5 mg twice-daily immediate-release TOF formulation, all patients had manifested inadequate therapeutic responses to methotrexate for at least 6 mo, having received a weekly dosage of up to 15 mg and at least one conventional synthetic disease-modifying anti-rheumatic drug (DMARD) at an adequate daily dosage. In addition, low-dose prednisolone was selectively prescribed (daily dosage of no more than 10 mg). Furthermore, patients were excluded from this study if they had previous exposure to targeted synthetic DMARDs treatment or were known to have diabetes, endocrine abnormalities, or critical medical disorders involving heart, lung, liver, and kidney.

Data collection and measurements

A detailed review was performed to collect data on the patients' demographic, clinical, laboratory and medication profiles. In addition to body mass index (BMI), clinical data included the 28-joint Disease Activity Score (DAS28) for RA activity[27], classifying as high (> 5.1), moderate (3.2-5.1) or low activity (2.6-3.2) and remission (< 2.6)[28]. Laboratory parameters included rheumatoid factor (RF)/anti-citrullinated peptide antibody (ACPA), C-reactive protein/erythrocyte sedimentation rate, and fasting blood levels of glucose and insulin. Seropositive RA was defined by the presence of either ACPA or RF. In addition to TOF, medication profiles were reviewed for use of prednisolone, conventional synthetic DMARDs with cyclosporin, hydroxychloroquine, leflunomide and sulfasalazine, and biologic synthetic DMARDs with abatacept, adalimumab (ADA), etanercept (ETA), golimumab (GOL), rituximab, and TCZ. For the calculation of IR, HOMA-IR, insulin (μU/mL) × glucose (mg/dL)/405, and Quantitative Insulin Sensitivity Check Index (QUICKI), 1/(log insulin (μU/mL) + glucose (mg/dL) were used in this study. The baseline HOMA-IR levels before TOF therapy were used to classify patients into high-IR (> 2.0) and low-IR (≤ 2.0) groups[7,29]. HOMA-IR and QUICKI measurements were obtained from all participants before and after a 24-wk therapeutic period. Furthermore, in the high-IR group, serial calculation data were available in selected patients before and after the TOF treatment.

Statistical analyses

Results were expressed as the mean ± SD. Serial HOMA-IR levels before and after starting TOF therapy were compared with the two-way analysis of variance with a post-hoc test. DAS28, HOMA-IR and QUICKI levels before and after a 24-wk therapeutic period were compared by using the Wilcoxon signed rank test. Different values and frequencies between high-IR and low-IR groups were compared using the Mann-Whitney and the chi-square/Fisher's exact tests, respectively. Spearman correlation coefficient test was used to correlate DAS28 values and HOMA-IR levels. A P value less than 0.05 was considered as significant in this study.

RESULTS

Baseline characteristics of active RA patients before TOF therapy

Fifty-six patients with 84% females and 88% seropositivity, aged 30 years to 75 years (52.3 ± 11.1 years), received TOF therapy for no less than 6 mo. They had BMI ranging from 19.2 kg/m^2 to 26.3 kg/m^2 (22.6 ± 2.0 kg/m^2), following the obesity definition of at least 27 kg/m^2 by the Ministry of Health and Welfare, Taiwan. Their DAS28 values varied from 4.54 to 7.37 (5.82 ± 0.74), all with moderate to high activity.

None had exposure to JAKi or succumbed to diabetes, endocrine or critical medical disorders involving major organs, fulfilling the selection criteria in this study.

Before the TOF treatment, 30 patients were naïve to biologic synthetic DMARDs therapy, and their DAS28 values varied from 5.16 to 7.37 (6.291 ± 0.530), all with high disease activity. Table 1 shows the demographic, clinical, laboratory and medication data for 30 naïve patients, classified into high- (n = 18) and low-IR (n = 12) groups according to their baseline HOMA-IR levels. There were no differences between high- and low-IR groups regarding age, sex, BMI, seropositivity and medication profile with prescription frequencies of various conventional synthetic DMARDs and low-dose prednisolone, as well as weekly methotrexate or daily/total prednisolone dosages. Before TOF therapy, there was a positive correlation between DAS28 values and HOMA-IR levels (r = 0.379, P = 0.039; Figure 1A), whereas a negative correlation was found between DAS28 values and QUICKI levels (r = -0.423, P = 0.020). Furthermore, higher DAS28 values were found in the high-IR group compared to the low-IR group (6.499 ± 0.472 *vs* 5.980 ± 0.470, P = 0.008), indicating that IR is driven by disease activity in RA patients[7, 30]. Notably, there were no changes in the patients' medication profiles during the 24-wk therapeutic period, with the exception of additional use of TOF.

In addition, 2 patients had an episode of single-dermatome herpes zoster (HZ) infection, both of which responded to valacyclovir therapy, with an incidence rate of 3.03 *per* 100 person-years. There is a general increased risk of HZ infection in RA patients[31], but especially in those receiving specific immunosuppressive therapy, including prednisolone (no less than 10 mg/d), methotrexate and anti-TNF-α biologics[32]. Interestingly, by analyzing health plan data from the United States, TOF-treated RA patients show an incidence rate of 3.87 *per* 100 person-years in HZ infection[33].

Effects of TOF therapy on IR in 30 active RA patients naïve to biologics

For 3 patients in the high-IR group, there were serial HOMA-IR calculations available for baseline at week 0 and after starting TOF therapy at weeks 4, 8, 12 and 24 (Figure 1B). In comparison with baseline levels, these patients who were naïve to biologics showed significantly lower levels only at week 24 but not at weeks 4, 8 or 12 (Figure 1B, week 0 *vs* weeks 24, 5.243 ± 0.571 *vs* 3.433 ± 0.664, P < 0.01). Further comparison with baseline HOMA-IR levels was carried out at week 24.

The levels of HOMA-IR and QUICKI before and after TOF therapy in the high-IR and the low-IR groups are shown in Table 2 and Figure 2. There were significantly reduced DAS28 values in both the high-IR and low-IR groups after the 24-wk TOF treatment (high-IR: 6.499 ± 0.472 *vs* 3.006 ± 0.445, P < 0.001; low-IR: 5.980 ± 0.470 *vs* 3.244 ± 0.614, P < 0.001). Significantly decreased HOMA-IR levels were found in the high-IR group (3.331 ± 1.036 *vs* 2.292 ± 0.707, P < 0.001; Figure 2B) but not in the low-IR group (1.602 ± 0.294 *vs* 1.430 ± 0.293, P = 0.139; Figure 2C), while significantly increased QUICKI levels were observed in the high-IR group (0.3207 ± 0.0135 *vs* 0.3397 ± 0.0154, P < 0.001; Figure 2E) but not in the low-IR group (0.3573 ± 0.0117 *vs* 0.3634 ± 0.0122, P = 0.156; Figure 2F). Furthermore, reduced HOMA-IR levels were observed in 17 patients in the high-IR group, while 7 patients in the low-IR group had a reduction in IR (high-IR *vs* low-IR: 94.4% *vs* 58.3%, P = 0.026). Despite observing no reduced IR after the TOF treatment in the low-IR group, a greater decrease in the values of DAS28 was found in 7 patients with decreased HOMA-IR levels, compared to 5 patients who showed no decrease (2.977 ± 0.237 *vs* 2.529 ± 0.362, P = 0.018), implicating reduced IR involvement in the responses to TOF therapy in active RA patients.

Effects of TOF therapy on IR in 26 active RA patients exposed to biologics

Before TOF therapy, 26 patients had been exposed to biologic synthetic DMARDs for at least 6 mo; the DMARDs included ADA, ETA, GOL and TCZ. This group of patients was consisted of 85% females, 89% with seropositivity, ages 40 years to 75 years (54.7 ± 10.6) and BMI 19.2 to 26.2 (22.96 ± 2.02). Their DAS28 values varied from 4.54 to 6.74 (5.265 ± 0.547), lower than that in those naïve to biologics (5.16 to 7.37, 6.291 ± 0.530, P < 0.001). The patients were divided into high- (n =19) and low-IR (n = 7) groups according to the baseline levels of HOMA-IR. All patients received methotrexate, while 5 patients in the high-IR group and 1 patient in the low-IR group received low-dose prednisolone therapy. No differences were found in the prescription frequencies of conventional synthetic DMARDs and low-dose prednisolone between two groups of patients.

The levels of HOMA-IR and QUICKI before and after TOF therapy in the high-IR and low-IR groups are shown in Table 3 and Figure 3. There were significantly reduced DAS28 values in both the high-IR and low-IR groups after the 24-wk TOF treatment (high-IR; 5.316 ± 0.807 *vs* 3.070 ± 0.466, P < 0.001; low-IR: 5.124 ± 0.470 *vs* 3.000 ± 0.672, P = 0.016). Significantly decreased HOMA-IR levels were found in the high-IR group (2.924 ± 0.790 *vs* 2.545 ± 1.080, P = 0.018; Figure 3B) but not in the low-IR group (1.527 ± 0.159 *vs* 1.453 ± 0.478, P = 0.781; Figure 3C), while significantly increased QUICKI levels were observed in the high-IR group (0.3273 ± 0.0117 *vs* 0.3372 ± 0.0214, P = 0.008; Figure 3E) but not the in low-IR group (0.3589 ± 0.0059 *vs* 0.3648 ± 0.0204, P = 0.813; Figure 2F).

Table 1 Baseline data of 30 active rheumatoid arthritis patients naïve to biologics

Group	All (*n* = 30)	High-IR (*n* = 18)	Low-IR (*n* = 12)	*P* value[1]
Sex (female %)	83.3	77.8	91.7	0.622
Age (yr)	50.3 ± 11.4 (30-74)	49.2 ± 10.5 (30-65)	51.8 ± 12.9 (31-74)	0.445
BMI (kg/m^2)	22.32 ± 1.93 (19.3-26.3)	22.56 ± 2.15 (19.7-26.3)	21.97 ± 1.57 (19.3-24.8)	0.624
Seropositivity (%)	86.7	83.3	91.7	0.632
DAS28	6.291 ± 0.530 (5.16-7.37)	6.499 ± 0.472 (5.56-7.37)	5.980 ± 0.470 (5.16-6.69)	0.008
ESR (mm/h)	51.7 ± 17.2 (26-88)	54.4 ± 18.5 (28-88)	47.6 ± 14.8 (28-70)	0.279
CRP (mg/L)	21.20 ± 6.90 (10.4-36.5)	22.27 ± 7.31 (10.4-36.5)	19.71 ± 6.21 (10.7-29.5)	0.341
Glucose (mg/dL)	88.7 ± 8.5 (66-104)	90.8 ± 9.3 (66-104)	85.6 ± 6.1 (77-98)	0.035
Insulin (μU/mL)	11.870 ± 5.029 (4.64-24.84)	14.710 ± 4.527 (9.07-24.84)	7.605 ± 1.410 (4.64-9.14)	< 0.001
HOMA-IR	2.639 ± 1.185 (1.07-5.89)	3.331 ± 1.036 (2.04-5.89)	1.602 ± 0.294 (1.07-2.00)	< 0.001
QUICKI	0.3353 ± 0.0222 (0.296-0.380)	0.3207 ± 0.0135 (0.296-0.343)	0.3573 ± 0.0117 (0.344-0.380)	< 0.001
Methotrexate (%)	100	100	100	1.0
Dosage (mg/wk)	15	15	15	1.0
Prednisolone (%)	26.7	22.2	33.3	0.678
Daily dosage[2] (mg/d)	5.6 ± 1.8	6.3 ± 2.5	5.0 ± 0.0	1.0
Total dosage[3] (mg)	865.6 ± 258.4	887.5 ± 386.5	843.8 ± 71.8	0.914
Hydroxychloroquine (%)	100	100	100	1.0
Sulfasalazine (%)	20.0	16.7	25.0	0.660
Leflunomide (%)	10.0	11.1	8.3	1.0

[1]High-IR *vs* Low-IR.
[2]Average daily prednisolone dosage in 1-mo period before enrolment into this study.
[3]Total exposure of prednisone dosages in 6-mo period before enrolment into this study.
HOMA-IR: Homeostatic model assessment-insulin resistance; QUICKI: Quantitative Insulin Sensitivity Check Index; BMI: Body mass index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; DAS28: Disease Activity Score 28.

DISCUSSION

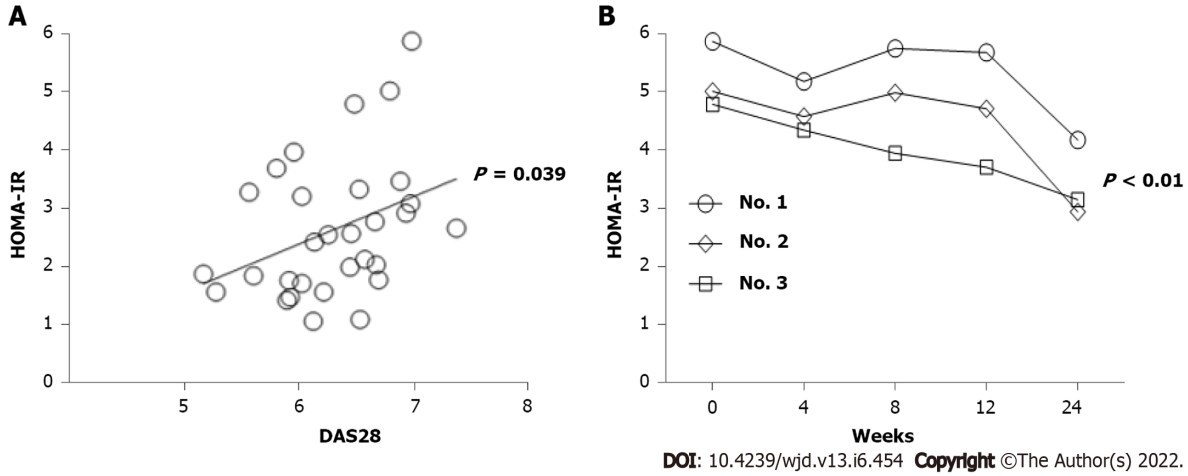
In this retrospective study, active RA patients receiving a 24-wk TOF treatment had significantly reduced IR among those with high baseline HOMA-IR levels. Furthermore, the clinical use of biologic synthetic DMARDs, including IL-6 and TNF-α blockers, has been demonstrated to reduce IR in non-diabetic active RA patients[22]. For patients with high IR before TOF therapy, baseline HOMA-IR levels were greater in those naïve to biologic agents than in those with an exposure history to anti-IL-6/TNF-α blocker (3.331 ± 1.036 *vs* 2.924 ± 0.790), while after therapy, there was a decrease in HOMA-IR levels with higher magnitude in naïve than exposed patients (31% *vs* 13% reduction, respectively). These results demonstrated, in this study, the effect of prescribed biologics on IR in active RA patients before TOF therapy. In addition to type 2 diabetes, IR is a crucial pathophysiological feature of obesity, with both conditions being characterized by persistent low-grade inflammation with increased levels of proinflammatory cytokines[34]. A reduction in IR has been identified in RA patients with a normal weight but not in those with obese status under anti-TNF-α therapy[35]. Despite no identified obesity in the present investigation (all patients had BMI < 27 kg/m^2), there were higher BMI levels for patients without IR reduction (n = 7) when compared to those with reduced IR (n = 30) in the high-IR group of patients naïve or exposed to biologic therapy (without *vs* with IR reduction: 24.53 ± 2.07 *vs* 22.49 ± 1.91 kg/m^2, P = 0.019), reflecting an influence of increased BMI on IR.

Recent investigations have indicated that when prescribed chronically, glucocorticoid (GC) can impair glucose tolerance and induce IR through stimulation of hepatic gluconeogenesis, alteration of insulin release from pancreatic β cells, and decrease in the sensitivity of the liver and muscle to insulin [36]. Since GC therapy is associated with a risk of developing type 2 diabetes, the EULAR recommends to wean RA patients off prednisolone use as early as possible[37]. Although methotrexate may enhance the actions of insulin on glucose transport and metabolism by increasing the extracellular concentration of adenosine, a retrospective study with 21340 RA patients under a 12-year follow-up demonstrated that the risk of type 2 diabetes was not lower with the use of methotrexate[38]. Hydroxychloroquine has

Table 2 Insulin resistance change in 30 active rheumatoid arthritis patients naïve to biologics by tofacitinib therapy

Group	Before	After	*P* value[1]
All (n = 30)			
DAS28	6.291 ± 0.530 (5.16-7.37)	3.101 ± 0.522 (2.08-4.21)	< 0.001
Decrease in DAS28		3.194 ± 0.609 (1.94-4.36)	
HOMA-IR	2.639 ± 1.185 (1.07-5.89)	1.947 ± 0.714 (0.98-4.19)	< 0.001
QUICKI	0.3353 ± 0.0222 (0.296-0.380)	0.3492 ± 0.0183 (0.310-0.385)	< 0.001
High IR (n = 18)			
DAS28	6.499 ± 0.472 (5.56-7.37)	3.006 ± 0.444 (2.52-4.21)	< 0.001
Decrease in DAS28		3.499 ± 0.536 (2.36-4.36)	
HOMA-IR	3.331 ± 1.036 (2.04-5.89)	2.292 ± 0.707 (1.21-4.19)	< 0.001
QUICKI	0.3207 ± 0.0135 (0.296-0.343)	0.3397 ± 0.0154 (0.310-0.372)	< 0.001
Low IR (n = 12)			
DAS28	5.980 ± 0.470 (5.16-6.69)	3.244 ± 0.614 (2.08-3.99)	< 0.001
Decrease in DAS28		2.736 ± 0.389 (1.94-3.23)	
HOMA-IR	1.602 ± 0.294 (1.07-2.00)	1.430 ± 0.293 (0.98-2.02)	0.139
QUICKI	0.3573 ± 0.0117 (0.344-0.380)	0.3634 ± 0.0122 (0.343-0.385)	0.156

[1]Before *vs* after TOF therapy.
HOMA-IR: Homeostatic model assessment-insulin resistance; QUICKI: Quantitative Insulin Sensitivity Check Index; DAS28: Disease Activity Score 28.



DOI: 10.4239/wjd.v13.i6.454

Figure 1 Characteristics of homeostatic model assessment-insulin resistance levels in active rheumatoid arthritis patients naïve to biologic synthetic disease-modifying anti-rheumatic drugs. A: Positive correlation between 28-joint disease activity score 28 values and homeostatic model assessment (HOMA)-insulin resistance (IR) levels (P = 0.039) before tofacitinib (TOF) therapy; B: Serial calculations of HOMA-IR levels in 3 patients with high baseline IR at weeks 0, 4, 8, 12 and 24 after TOF therapy. There were significantly lower levels at week 24 as compared with those at week 0 (P < 0.01). HOMA-TR: Homeostatic model assessment-insulin resistance.

beneficial effects on the release and sensitivity of insulin, and a multicenter prospective study with 4950 RA patients showed a lower risk of developing type 2 diabetes in those receiving hydroxychloroquine treatment[39]. In this study, only 14 patients (25%) received low-dose prednisolone prescription before TOF therapy, and most of them (86%) had reduced HOMA-IR levels after therapy. Furthermore, there were no differences in the prescription frequencies and the dosages of various conventional synthetic DMARDs between the two patient groups with different baseline IRs, and their medication profiles were stable throughout the therapeutic period. In the present investigation, the effects of 24-wk TOF therapy on IR reduction could be identified in RA patients with high baseline DAS28 values and HOMA-IR levels. Notably, reduced IR in active RA only with high baseline IR has been demonstrated by studies with IR classification occurring before anti-IL-6 or anti-TNF-α therapy[7,11,35,40-42].

Table 3 Insulin resistance change in 26 active rheumatoid arthritis patients exposed to biologics by tofacitinib therapy

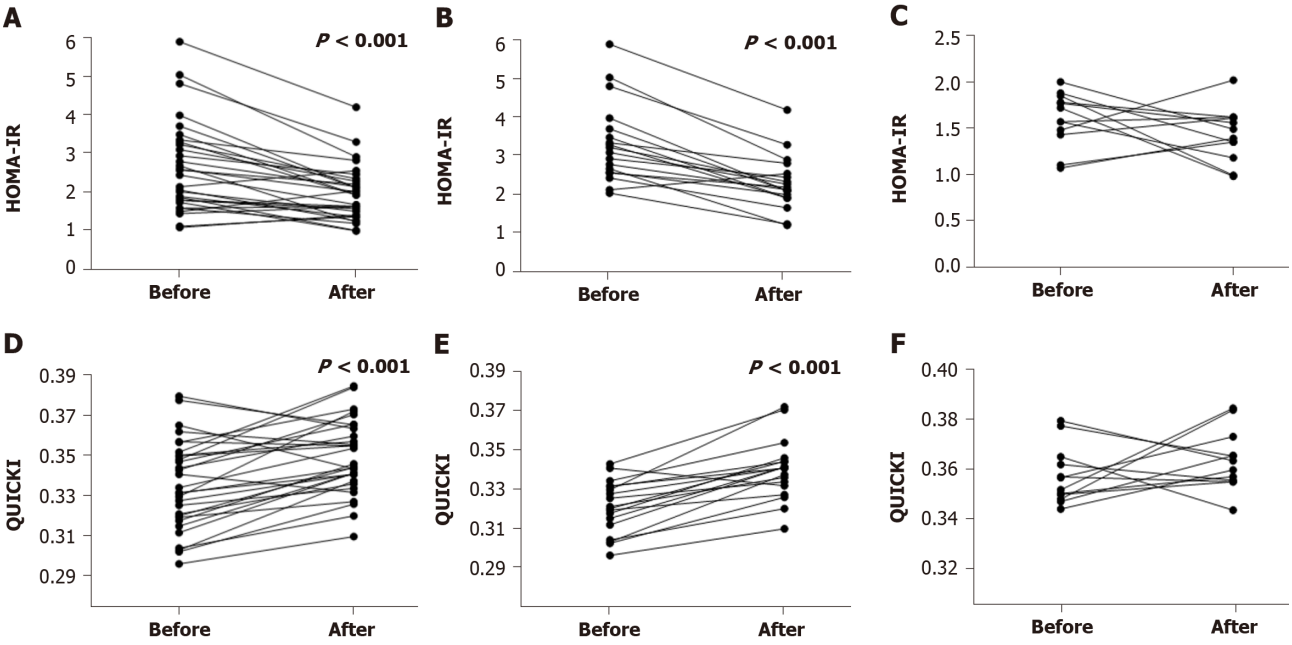
Group	Before	After	*P* value[1]
All (n = 26)			
DAS28	5.265 ± 0.547 (4.54-6.74)	3.051 ± 0.516 (2.11-3.99)	< 0.001
Decrease in DAS28		2.214 ± 0.688 (1.08-3.49)	
HOMA-IR	2.548 ± 0.925 (1.33-4.75)	2.251 ± 1.067 (0.85-4.55)	0.016
QUICKI	0.3358 ± 0.0177 (0.305-0.366)	0.3446 ± 0.0242 (0.305-0.394)	0.016
High IR (n = 19)			
DAS28	5.316 ± 0.807 (4.63-6.74)	3.070 ± 0.466 (2.42-3.90)	< 0.001
Decrease in DAS28		2.246 ± 0.672 (1.08-3.49)	
HOMA-IR	2.924 ± 0.790 (2.10-4.75)	2.545 ± 1.080 (1.05-4.55)	0.018
QUICKI	0.3273 ± 0.0117 (0.305-0.341)	0.3372 ± 0.0214 (0.305-0.380)	0.008
Low IR (n = 7)			
DAS28	5.124 ± 0.332 (4.54-5.48)	3.000 ± 0.672 (2.11-3.99)	0.016
Decrease in DAS28		2.124 ± 0.778 (1.25-3.33)	
HOMA-IR	1.527 ± 0.159 (1.33-1.77)	1.453± 0.478 (0.85-2.18)	0.781
QUICKI	0.3589 ± 0.0059 (0.350-0.366)	0.3648 ± 0.0204 (0.340-0.394)	0.813

[1]Before *vs* after TOF therapy.
HOMA-IR: Homeostatic model assessment-insulin resistance; QUICKI: Quantitative Insulin Sensitivity Check Index; TOF: Tofacitinib; DAS28: Disease Activity Score 28.

Accumulated evidence has indicated that the JAK-STAT pathway is required for normal homeostasis of metabolic processes, and when it is dysregulated it contributes to the development of obesity and diabetes type 2 associated with chronic low-grade inflammatory response[43]. Numerous investigations have found the involvement of JAK-STAT signaling in peripheral metabolic organs with adipose, liver, muscle and pancreas, and in diabetes types 1 and 2[44]. A crucial role of JAK signaling, involving JAK2 in particular, has been recognized in regulating metabolic processes with glucose tolerance, insulin sensitivity and adiposity through studies using conditional genetic ablation mouse models. Mice with hepatocyte-specific deletion of JAK2 had reduced adiposity, increased pancreatic β-cell mass and complete protection against high-fat diet (HFD)-induced IR and glucose intolerance[45]. Mice with adipocyte-specific loss of JAK2 showed increased insulin sensitivity and resistance to HFD-induced metabolic inflammation[46]. Furthermore, besides an involvement in the activation of cytokine signaling pathways, the JAK-STAT pathway has been shown to regulate the function and survival of the β cells [43,44]. In the non-obese diabetic mouse model, disruption of STAT1 could inhibit interferon-γ-induced β cell apoptosis[47], while treating mice with a JAK1/JAK2 inhibitor reversed diabetes through blockade of the MHC class I upregulation on β cells[48]. Notably, experiments with diabetic animal models have demonstrated that systemic administration of TOF, a pan-JAKi, could normalize impaired glucose tolerance and insulin response in Lnk deficient mice, and reduce IR and improve β-cell function in fructose/streptozotocin-induced rats[49,50]. In this clinical study, oral TOF therapy showed a beneficent effect on IR reduction in active RA patients. In sum, these findings implicate JAK-STAT signaling as a pharmacological target in diabetes and the potential for JAKi use in treating diabetic patients.

CONCLUSION

In this retrospective study, we observed a reduction of IR following 24-wk TOF therapy with 5 mg twice-daily immediate-release formulation in non-diabetic RA patients with active disease. Further prospective studies can be performed in both non-diabetic patients and those with comorbid diabetes to clearly elucidate the effect of TOF on IR in active RA.



DOI: 10.4239/wjd.v13.i6.454

Figure 2 Homeostatic model assessment-insulin resistance and Quantitative Insulin Sensitivity Check Index levels in 30 active rheumatoid arthritis patients naïve to biologic agents before and 24 wk after tofacitinib therapy. A: Homeostatic model assessment (HOMA)-insulin resistance (IR) levels in all 30 patients at weeks 0 and 24 after tofacitinib (TOF) therapy ($P < 0.001$); B: HOMA-IR levels in the high-IR group with 18 patients at weeks 0 and 24 after TOF therapy ($P < 0.001$); C: HOMA-IR levels in the low-IR group with 12 patients at weeks 0 and 24 after TOF therapy; D: Quantitative Insulin Sensitivity Check Index (QUICKI) levels in all 30 patients at weeks 0 and 24 after TOF therapy ($P < 0.001$); E: QUICKI levels in the high-IR group with 18 patients at weeks 0 and 24 after TOF therapy ($P < 0.001$); F: QUICKI levels in the low-IR group with 12 patients at weeks 0 and 24 after TOF therapy. QUICKI: Quantitative Insulin Sensitivity Check Index; HOMA-TR: Homeostatic model assessment-insulin resistance.

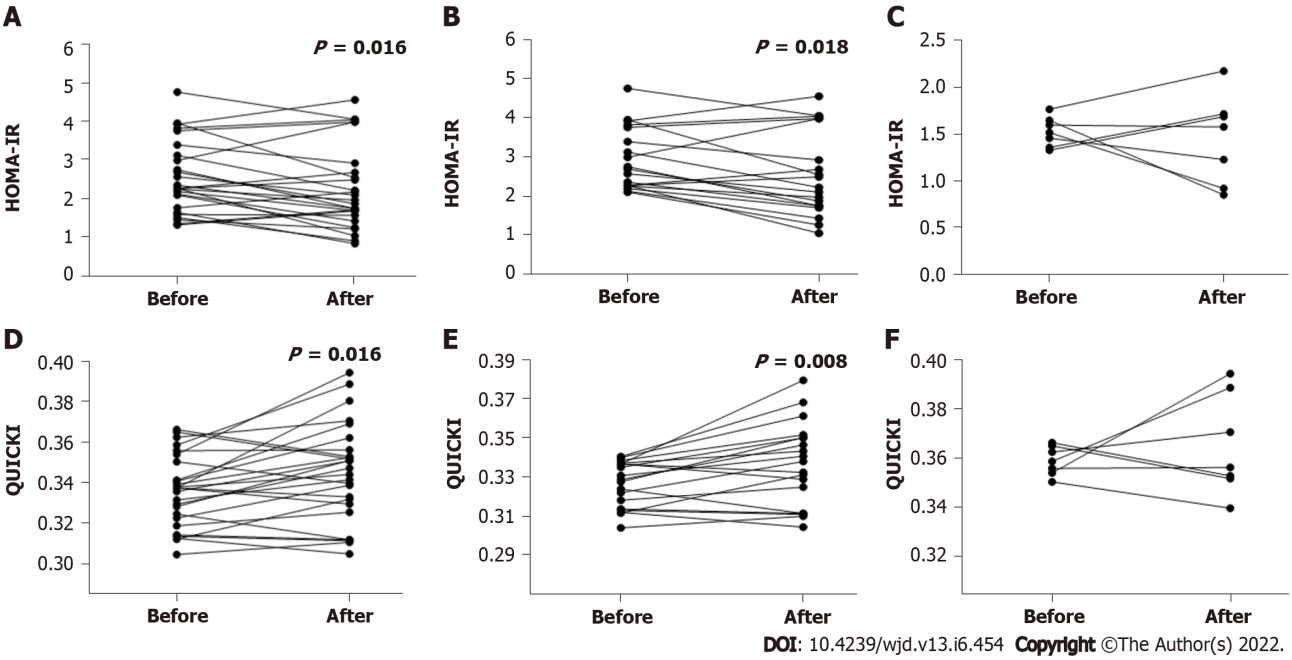


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Figure 3 Homeostatic model assessment-insulin resistance and Quantitative Insulin Sensitivity Check Index levels in 26 active rheumatoid arthritis patients exposed to biologic agents before and 24 wk after tofacitinib therapy. A: Homeostatic model assessment (HOMA)-insulin resistance (IR) levels in all 26 patients at weeks 0 and 24 after tofacitinib (TOF) therapy ($P = 0.016$); B: HOMA-IR levels in the high-IR group with 19 patients at weeks 0 and 24 after TOF therapy ($P = 0.018$); C: HOMA-IR levels in the low-IR group with 7 patients at weeks 0 and 24 after TOF therapy; D: Quantitative Insulin Sensitivity Check Index (QUICKI) levels in all 26 patients at weeks 0 and 24 after TOF therapy ($P = 0.016$); E: QUICKI levels in the high-IR group with 19 patients at weeks 0 and 24 after TOF therapy ($P = 0.008$); F: QUICKI levels in the low-IR group with 7 patients at weeks 0 and 24 after TOF therapy. QUICKI: Quantitative Insulin Sensitivity Check Index; HOMA-TR: Homeostatic model assessment-insulin resistance.

ARTICLE HIGHLIGHTS

Research background

An increased risk of insulin resistance (IR) has been identified in rheumatoid arthritis (RA), a chronic inflammatory disorder with elevated levels of pathogenic cytokines. Biologics targeting proinflammatory cytokines can control the disease and improve insulin sensitivity in RA.

Research motivation

Although Janus kinase (JAK) signaling can regulate cytokine receptors and participate in RA pathogenesis, it remains to be elucidated whether there is a reduction of IR in such patients under JAK inhibitor (JAKi) therapy.

Research objectives

This study examined the effect of JAKi treatment on the reduction of IR in RA with active disease.

Research methods

A retrospective study was carried out in non-diabetic active RA patients under tofacitinib (TOF) therapy with 5 mg twice-daily immediate-release formulation from 2017 to 2021.

Research results

Fifty-six RA patients aged 30 years to 75 years (52.3 ± 11.1) with DAS 28 values 4.54 to 7.37 (5.82 ± 0.74), were classified into high- and low-IR groups based on the baseline homeostatic model assessment (HOMA)-IR levels. For the 30 patients naive to biologics, after a 24-wk therapeutic period, reduced levels of HOMA-IR were observed in the high-IR group (3.331 ± 1.036 *vs* 2.292 ± 0.707, $P < 0.001$). In another 26 patients exposed to tumor necrosis factor-α or interleukin-6 blockers, despite showing a decrease with lower magnitude than that observed in the naïve patients, reduced HOMA-IR levels were also identified in the high-IR group (2.924 ± 0.790 *vs* 2.545 ± 1.080, $P = 0.018$).

Research conclusions

In this retrospective study, our results demonstrated reduced IR following 24-wk TOF therapy in non-diabetic active RA patients.

Research perspectives

Further prospective studies can be performed in both non-diabetic patients and those with comorbid diabetes to clearly elucidate the effect of TOF on IR in active RA.

ACKNOWLEDGEMENTS

The authors are indebted to all of the doctors/nurses involved in the management of RA patients at the National Cheng Kung University Hospital (NCKUH). The statistical analyses of this study were performed with help from the Biostatistics Consulting Center at the NCKUH.

FOOTNOTES

Author contributions: Wang CR designed the report, collected the clinical data, and wrote the paper; Wang CR and Tsai HW analyzed the clinical data.

Institutional review board statement: This study was approved by the Ethics Committee of National Cheng Kung University Hospital, No. B-ER-105-108.

Informed consent statement: The Institutional Review Board waived the requirement of informed consent from each patient due to the study being classified as a retrospective review of medical records.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The data of this study can be provided to researchers by the corresponding author upon reasonable request.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 June 15; 13(6): 466-470

DOI: 10.4239/wjd.v13.i6.466

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Relook at DPP-4 inhibitors in the era of SGLT-2 inhibitors

Awadhesh Kumar Singh, Ritu Singh

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Li SY, China; tong WN, China
A-Editor: Lin FY, China

Received: March 7, 2022
Peer-review started: March 7, 2022
First decision: April 17, 2022
Revised: April 19, 2022
Accepted: May 21, 2022
Article in press: May 21, 2022
Published online: June 15, 2022

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Abstract

SGLT-2 inhibitors (SGLT-2Is) have significantly improved cardio-renal outcomes and are preferred agents in people with cardiovascular diseases, heart failure, and diabetic kidney disease. Similarly, GLP-1 receptor agonists (GLP-1RAs) have significantly improved atherosclerotic cardiovascular outcomes. To this end, DPP-4 inhibitors (DPP-4Is) are cardiac-neutral drugs. While long-acting GLP-1RAs have shown a favorable HbA1c lowering compared to DPP-4Is, there is no clinically meaningful HbA1c lowering difference between SGLT-2Is *vs* DPP-4Is. Moreover, the glucose-lowering potential of SGLT-2Is gets compromised with a progressive decline in renal functions, unlike DPP-4Is. Furthermore, the HbA1c lowering potential of DPP-4Is is favorable in people with T2DM having a modest baseline HbA1c (8.0%-8.5%) compared with SGLT-2Is which lowers HbA1c larger in a background of higher baseline HbA1c (> 8.5%-9.0%). These findings suggest that the role of DPP-4Is in the management of type 2 diabetes mellitus cannot be completely ignored even in the era of SGLT-2Is.

Key Words: DPP-4 inhibitors; SGLT-2 inhibitors; GLP-1 receptor agonists; Cardiovascular outcomes; Renal outcomes

Core Tip: Despite the newer anti-diabetic agents such as SGLT-2 inhibitors (SGLT-2Is) and GLP-1 receptor agonists have taken the center stage in the management of type 2 diabetes mellitus due to additional cardiac and renal benefits, the role of DPP-4 inhibitors (DPP-4Is) cannot be undermined. HbA1c lowering potential of DPP-4Is are nearly similar to SGLT-2Is and surprisingly larger in a background of modest baseline HbA1c compared with SGLT-2Is. Moreover, the HbA1c lowering abilities of SGLT-2Is are compromised with declining renal function while DPP-4Is reduce HbA1c favorably in people with chronic kidney disease regardless of impaired kidney functions.

Citation: Singh AK, Singh R. Relook at DPP-4 inhibitors in the era of SGLT-2 inhibitors. *World J Diabetes* 2022; 13(6): 466-470
URL: https://www.wjgnet.com/1948-9358/full/v13/i6/466.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i6.466

TO THE EDITOR

We read with interest a minireview by Florentin *et al*[1] putting their arguments in favor of DPP-4 inhibitors (DPP-4Is) as a second-line drug after metformin in people with type 2 diabetes mellitus (T2DM) in particular who are elderly and have chronic kidney disease (CKD) stage 3A or lower. This wonderfully written minireview discusses the role of DPP-4Is in the era of two other novel anti-diabetic agents such as SGLT-2 inhibitors (SGLT-2Is) and GLP-1 receptor agonists (GLP-1RAs) that have shown a remarkably beneficial effect on cardiovascular (CV) and renal endpoints making them an ideal second or arguably even first-line drug in people with T2DM having established CV disease (CVD), heart failure (HF) and CKD. While authors have discussed the pharmacological differences amongst different DPP-4Is and put a perspective on the CV outcome trials in the era of SGLT-2Is and GLP-1RAs, few vital details seem to be missing and some of the statements appear rather ambiguous that need clarification. The most important area that is surprisingly missing in this review is the efficacy comparison between DPP-4Is *vs* SGLT-2Is or GLP-1RAs. Expectedly, the HbA1c lowering effect of DPP-4Is would be inferior to GLP-1RAs owing to their mechanism of action that causes a physiological *vs* pharmacological rise of GLP-1 respectively and indeed, several head-to-head studies of long-acting GLP-1RAs have shown a superior HbA1c lowering beside a significant reduction in weight and systolic blood pressure (SBP) when compared with DPP-4Is. However, the HbA1c lowering effect of DPP-4Is is not clinically meaningful different from SGLT-2Is. To this end, several studies have evaluated the HbA1c lowering effect of SGLT-2Is *vs* DPP4Is in the past decade[2-8]. Although in most of these SGLT-2Is head-to-head studies with DPP-4Is, HbA1c reduction was similar between the two drug classes; DPP-4Is were used as an open-label active comparator arm only for exploratory analysis. One study that compared empagliflozin 10 and 25 mg with 100 mg sitagliptin as an active comparator in a double-blind randomized fashion found no difference in HbA1c lowering[3]. However, two studies that compared canagliflozin 100 and 300 mg with sitagliptin 100 mg as an active comparator in a double-blind randomized fashion, found 300 mg canagliflozin to be superior to 100 mg sitagliptin in HbA1c lowering, though no difference was noted with 100 mg canagliflozin (Table 1)[6,7]. Meta-analyses that compared HbA1c lowering with DPP-4Is *vs* SGLT-2Is yielded discordant results[9-12]. While some found no difference in HbA1c lowering, others showed a small but significant HbA1c lowering with SGLT-2Is compared to DPP-4Is (Table 1). Notably, weight and SBP reduction were consistently superior with SGLT-2I *vs* DPP-4I in all these head-to-head studies including meta-analyses. Another interesting piece of missing information that needs discussion is the differential HbA1c lowering effect of DPP-4Is vs. SGLT-2Is stratified on baseline HbA1c. While the SGLT-2Is appear to lower the HbA1c more favorably compared with DPP-4Is in the background of higher baseline value (HbA1c 8.5%-9.0%), DPP-4Is lowered HbA1c more favorably compared with SGLT-2I in people having a modest baseline HbA1c value (< 8%-8.5%) (Table 1)[13-15]. This finding suggests DPP-4Is may have a favorable effect on HbA1c lowering compared to SGLT-2Is in people with T2DM having a modest baseline HbA1c, in absence of high CV risk. Although a reduction in HbA1c is always larger when baseline HbA1c is high, we do not know exactly why DPP-4Is reduce HbA1c larger compared to the SGLT-2Is when the baseline value is modest. Since SGLT-2Is HbA1c lowering ability is dependent on the renal threshold of glucose excretion (RT_G), modest baseline HbA1c may not produce further lowering of RT_G.

Nevertheless, we humbly disagree with the author's conclusion about "the lack of evidence with SGLT-2Is and GLP-1RAs in elderly patients with diabetes as well as the contraindication of SGLT-2Is in patients with CKD, grade 3A and lower, make DPP-4Is a safe choice in such populations." Let us recall that: (1) About one-fourth patients population (24.2%) in HF trial of SGLT-2I dapagliflozin were elderly [≥ 75 years, median age 79 years (76-82 years)] and they benefitted equally [Hazard ratio (HR), 0.68; 95% Confidence interval (CI), 0.53-0.88] when compared to the overall population (HR, 0.74; 95%CI, 0.65-0.85) in terms of reduction of the primary composite endpoint of CV death or HF hospitalization (HHF) or urgent HF visits ($P_{interaction}$ = 0.76)[16]; (2) Mean age of the population in CV-, HF- and renal-outcome trials of SGLT-2Is varied from as low as 62 years in renal outcome trial of dapagliflozin (DAPA-CKD) to as high as 72 years in HF trial of empagliflozin (EMPEROR-Preserved) that found a significantly beneficial renal and CV effect respectively[17]; (3) Current guidelines recommend using SGLT-2Is in patients with CKD if eGFR is ≥ 30 mL/min/1.73 m^2 and in addition, empagliflozin has been granted an additional label of use up to eGFR ≥ 20 mL/min/m^2 in patients with HF with reduced ejection fraction and CKD[18]; (4) The latest Kidney Disease: Improving Global Outcomes 2022 guideline which is currently under public review recommend using SGLT-2Is in patients with CKD if eGFR ≥ 20 mL/min/1.73 m^2 regardless of background HF. Moreover, once SGLT-2Is are initiated it is reasonable to continue even if the eGFR falls below 20 mL/min per 1.73 m^2 unless it is not tolerated or kidney

Table 1 HbA1c reduction with SGLT-2 inhibitors *vs* DPP-4 inhibitors

Ref.	Study duration (wk)	Background therapy	*n* (Active drug)	Baseline HbA1c	SGLT-2I (A) (% HbA1c reduction)	DPP-4I (B) (% HbA1c reduction)	Δ A minus B (95%CI)
HbA1c reduction with SGLT-2Is *vs* DPP-4Is in head-to-head randomized controlled trials							
Rosenstock *et al*[2], 2012	12	Metformin	193	7.6%-7.8%	-0.76 (Cana 100 mg) -0.92 (Cana 300 mg)	-0.74 (Sita 100 mg)	NC, (B) exploratory
Roden *et al*[3], 2013	24	Drug naïve	671	7.9%	-0.66 (Empa 10 mg) -0.78 (Empa 25 mg)	-0.66 (Sita 100 mg)	0.0 (-0.15, 0.14) -0.12 (-0.26, 0.03)
Rosenstock *et al*[4], 2013	12	Metformin	212	7.9%-8.1%	-0.56 (Empa 10 mg) -0.55 (Empa 25 mg)	-0.45 (Sita 100 mg)	NC, (B) exploratory
Ferrannini *et al*[5], 2013	90	Metformin	332	7.9%-8%	-0.34 (Empa 10 mg) -0.63 (Empa 25 mg)	-0.40 (Sita 100 mg)	NC, (B) exploratory
Lavalle-González *et al*[6], 2013	52	Metformin	1079	7.9%	-0.73 (Cana 100 mg) -0.88 (Cana 300 mg[a])	-0.73 (Sita 100 mg)	-0.15[a] (-0.27, -0.03)
Schernthaner *et al*[7], 2013	52	Metformin + SU	755	8.1%	-1.03 (Cana 300 mg[a])	-0.66 (Sita 100 mg)	-0.37[a] (-0.50, -0.25)
Amin *et al*[8], 2015	12	Metformin	328	8.1%	-0.80 (Ertu 5 mg)	-0.87 (Sita 100 mg)	NC, (B) exploratory
Difference in HbA1c reduction with SGLT-2Is *vs* DPP-4Is in meta-analyses							
Pinto *et al*[9], 2015	≥ 12	LSM, Metformin, SU	NR (6 studies)	-	SGLT-2Is	DPP-4Is	-0.15[a] (-0.21, -0.08)
Maruthur *et al*[10], 2016	≤ 52	Metformin	1278 (4 studies)	-	SGLT-2Is	DPP-4Is	(B) minus (A) = +0.17[a] (0.08, 0.26)
Wang *et al*[11], 2018	12-78	Metformin	3454 (7 studies)	-	SGLT-2Is	DPP-4Is	(B) minus (A) = +0.11 (-0.03, 0.25)
Mishriky *et al*[12], 2018	≤ 26	Metformin	2462 (6 studies)	-	SGLT-2Is	DPP-4Is	(B) minus (A) = +0.05 (-0.05, 0.16)
	≥ 52	Metformin	1872 (3 studies)	-	SGLT-2Is	DPP-4Is	(B) minus (A) = +0.11[a] (0.03, 0.20)
HbA1c reduction with SGLT-2Is *vs* DPP-4Is in head-to-head randomized controlled trial stratified on baseline HbA1c							
Rosenstock *et al*[13], 2015	24	Metformin	190	> 9%	-1.87 (Dapa 10 mg)	-1.32 (Saxa 5 mg)	NC
			103	< 8%	-0.45 (Dapa 10 mg)	-0.69 (Saxa 5 mg)	
Lewin *et al*[14], 2015	24	LSM	116	≥ 8.5%	-1.66 (Empa 25 mg) -1.54 (Empa 10 mg	-1.07 (Lina 5 mg)	NC
			473	< 8.5%	-0.66 (Empa 25 mg) -0.56 (Empa 10 mg)	-0.55 (Lina 5 mg)	NC
DeFronzo *et al*[15], 2015	24	Metformin	101	≥ 8.5%	-1.22 (Empa 25 mg) -1.29 (Empa 10 mg)	-0.99 (Lina 5 mg)	NC
			508	< 8.5%	-0.43 (Empa 25 mg) -0.46 (Empa 10 mg)	-0.62 (Lina 5 mg)	NC

[a](A) superior over (B).

SGLT-2Is: SGLT-2 inhibitors; DPP-4Is: DPP4 inhibitors; Cana: Canagliflozin; Empa: Empagliflozin; Dapa: Dapagliflozin; Ertu: Ertugliflozin; Sita: Sitagliptin; Saxa: Saxagliptin; Lina: Linagliptin; SU: Sulfonylureas; LSM: Life style modification; NC: Not compared.

replacement therapy is initiated[19]; (5) Although there are no head-to-head randomized controlled trials that compared CV outcomes between DPP-4Is *vs* DPP-4Is, several large real-world, propensity-matched studies showed a significant reduction in HHF with SGLT-2Is compared with DPP-4Is in patients with T2DM, regardless of baseline high CV risk[20]; and (6) Finally, the 2011 European Diabetes Working Party for Older People clinical guideline that recommended DPP-4I as a second-line drug of choice in elderly were made before the US Federal Drug Administration approval of first SGLT-2I

canagliflozin in 2013 and first positive CV outcome with empagliflozin in 2015. These findings suggest author's conclusion is discordant with the available evidence[21].

FOOTNOTES

Author contributions: Singh AK designed the research; Singh R performed the research, Singh AK and Singh R analyzed the data; Singh AK wrote the letter, and Singh R revised the manuscript.

Conflict-of-interest statement: The authors have nothing to disclose.

Country/Territory of origin: India

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com

ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 July 15; 13(7): 471-586

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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Monthly Volume 13 Number 7 July 15, 2022

World Journal of Diabetes

Monthly Volume 13 Number 7 July 15, 2022

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJD* as 4.560; IF without journal self cites: 4.450; 5-year IF: 5.370; Journal Citation Indicator: 0.62; Ranking: 62 among 146 journals in endocrinology and metabolism; and Quartile category: Q2.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

PUBLICATION DATE
July 15, 2022

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 471-481

DOI: 10.4239/wjd.v13.i7.471

ISSN 1948-9358 (online)

EDITORIAL

Renin-angiotensin system blockers-SGLT2 inhibitors-mineralocorticoid receptor antagonists in diabetic kidney disease: A tale of the past two decades!

Awadhesh Kumar Singh, Ritu Singh

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Techane T; Wang QY, China

Received: March 11, 2022
Peer-review started: March 11, 2022
First decision: April 18, 2022
Revised: April 19, 2022
Accepted: June 18, 2022
Article in press: June 18, 2022
Published online: July 15, 2022

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Abstract

Several pharmacological agents to prevent the progression of diabetic kidney disease (DKD) have been tested in patients with type 2 diabetes mellitus (T2DM) in the past two decades. With the exception of renin-angiotensin system blockers that have shown a significant reduction in the progression of DKD in 2001, no other pharmacological agent tested in the past two decades have shown any clinically meaningful result. Recently, the sodium-glucose cotransporter-2 inhibitor (SGLT-2i), canagliflozin, has shown a significant reduction in the composite of hard renal and cardiovascular (CV) endpoints including progression of end-stage kidney disease in patients with DKD with T2DM at the top of renin-angiotensin system blocker use. Another SGLT-2i, dapagliflozin, has also shown a significant reduction in the composite of renal and CV endpoints including death in patients with chronic kidney disease (CKD), regardless of T2DM status. Similar positive findings on renal outcomes were recently reported as a top-line result of the empagliflozin trial in patients with CKD regardless of T2DM. However, the full results of this trial have not yet been published. While the use of older steroidal mineralocorticoid receptor antagonists (MRAs) such as spironolactone in DKD is associated with a significant reduction in albuminuria outcomes, a novel non-steroidal MRA finerenone has additionally shown a significant reduction in the composite of hard renal and CV endpoints in patients with DKD and T2DM, with reasonably acceptable side effects.

Key Words: Renin-angiotensin system blockers; SGLT-2 inhibitors; Mineralocorticoid receptor antagonist; Diabetic kidney disease; Chronic kidney disease; Renal outcomes; Cardiovascular outcomes

Core Tip: Angiotensin receptor blockers were the first drug class to show a conclusive benefit in preventing diabetic kidney disease (DKD) progression through two randomized trials IDNT and RENAAL in 2001. Several newer pharmacological agents have been tested in DKD in the past 20 years without much success. Notably, recently conducted renal outcome trials of sodium-glucose cotransporter-2 inhibitors in patients with DKD such as CREDENCE, DAPA-CKD, and EMPA-KIDNEY have shown significant improvement in disease progression. Similarly, recent trials of the non-steroidal mineralocorticoid receptor antagonist finerenone (FIDELIO-DKD and FIGARO-DKD) have shown significant improvement in both renal and cardiac endpoints in DKD.

Citation: Singh AK, Singh R. Renin-angiotensin system blockers-SGLT2 inhibitors-mineralocorticoid receptor antagonists in diabetic kidney disease: A tale of the past two decades!. *World J Diabetes* 2022; 13(7): 471-481
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/471.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.471

INTRODUCTION

Type 2 diabetes mellitus (T2DM) remains the leading cause of both chronic kidney disease (CKD) and end-stage kidney disease (ESKD) worldwide[1]. The exact incidence and prevalence of CKD and ESKD from T2DM is difficult to assess due to infrequently performed invasive procedure of kidney biopsies (the gold standard for diagnosis of diabetic kidney disease [DKD]); and because most patients with DKD die before requiring renal replacement therapy. However, DKD affects nearly 20% of patients with T2DM[2-4]. Several factors that may lead to DKD include: the formation of advanced glycation end-products; generation of reactive oxygen species; activation of intercellular signals for proinflammatory and profibrotic gene expression causing cellular inflammation, injury, and fibrosis; alterations in glomerular hemodynamics; and associated hyperinsulinemia and insulin resistance further activating these pathogenic mechanisms[5]. Although the time to development of DKD in T2DM depends on multiple risk factors, its incidence is about 2% of patients per year and affects nearly 25% of patients within 10 years of diagnosis[6]. Classically, DKD progresses from three stages of albuminuria based on urinary albumin excretion: normal to mildly increased (< 30 mg/d or albumin/creatinine ratio [ACR] of < 30 mg/g), moderately increased (formerly called microalbuminuria-30 to 300 mg per day or ACR 30-300 mg/g), and severely increased (formerly called macroalbuminuria-> 300 mg per day or ACR > 300 mg/g) albuminuria. Importantly, the presence of severe albuminuria increases the annual risk of mortality by 4.6% compared with the risk of progression to ESKD (by 2.3%)[6]. These findings necessitate the role of pharmacological agents other than glycemic control in the management of DKD in patients with T2DM.

MANAGEMENT OF DKD IN T2DM

The general approach to managing DKD is similar to that in all patients with T2DM, which includes smoking cessation, weight loss, regular exercise, individualized glycemic targets, and statins. However, certain specific considerations are additionally needed in DKD which include: more intensive blood pressure lowering to prevent ESKD and cardiovascular (CV) morbidity in patients with severe albuminuria and to reduce mortality; and mandatory use of renin-angiotensin system blockers (RASBs), either angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), but not both. Since most individuals with DKD and hypertension require combination therapy, either a combination of an ACEI or ARB plus a dihydropyridine calcium channel blocker is the preferred regimen, except in patients with severe albuminuria where either a non-dihydropyridine CCB or a diuretic may be more suitable with RASB[7].

RASB era

Although there are several randomized controlled trials (*e.g.*, landmark studies: MICRO-HOPE, IRMA-2, and ADVANCE), which showed that RASB prevented progression from normal to microalbuminuria and micro- to macro-albuminuria in T2DM, reduction of albuminuria has generally been considered only a soft renal surrogate endpoint[8-10]. The first convincing evidence suggesting that RASB can significantly reduce hard renal endpoints and prevent the progression of CKD to ESKD in patients with T2DM with severe albuminuria dates back to 2001. The Irbesartan Diabetic Nephropathy Trial (IDNT) randomized 1715 T2DM patients (having urine protein excretion ≥ 0.9 g/d and mean serum creatinine of 1.7 mg/dL) to either irbesartan or amlodipine or placebo. At 2.6 years, the primary composite renal outcome (doubling of serum creatinine, development of ESKD or death from any cause) with irbesartan

was 20% lower than placebo (hazard ratio [HR], 0.80; 95% confidence interval [CI]: 0.66-0.97; P = 0.02) and 23% lower than amlodipine (HR: 0.77; 95%CI: 0.63-0.93; P = 0.006). However, neither any significant reduction in secondary CV endpoint (CV death, non-fatal myocardial infarction [MI], non-fatal stroke, heart failure hospitalization [HHF], or lower limb amputation) nor any reduction in all-cause death was noted with irbesartan, compared to either placebo or amlodipine[11]. The Reduction of Endpoints in Non-Insulin-Dependent Diabetes Mellitus with the Angiotensin II Antagonist Losartan (RENAAL) trial randomized 1513 T2DM patients (having albuminuria > 300 mg/d and mean serum creatinine of 1.9 mg/dL) to either losartan or placebo or both, in addition to conventional antihypertensive drugs (but not ACEI). At 3.4 years, the primary outcome (doubling of serum creatinine, development of ESKD, or death from any cause) was reduced by 16% (HR: 0.84; 95%CI: 0.72-0.98; P = 0.020) in losartan *vs* the placebo group. However, no reduction in all-cause death was noted between losartan *vs* placebo[12]. Importantly, despite the positive renal outcomes with ARBs, a substantial residual risk did remain in both IDNT (residual risk-32.6%) and RENAAL trials (residual risk-43.5%). These findings necessitate additional safe pharmacological agents along with RASBs to further reduce the remaining residual risks in patients with DKD.

Experimental combination therapy and novel drug era

From 2001 until 2018, several combinations of RASB (ACEI plus ARB such as lisinopril plus losartan [VA NEPHRON-D trial] or telmisartan plus ramipril [ONTARGET trial]) were tried without any success. Few older agents such as atorvastatin (4D trial) and several newer novel pharmacological agents (*e.g.*, protein kinase C β [PKC-β] inhibitor: ruboxistaurin; darbepoetin-alfa; non-selective endothelin A receptor antagonist: avosentan; tumor growth factor-β [TGF-β] inhibitor: pirfenidine; pyridoxamine; a mixture of natural glycosaminoglycans polysaccharide: sulodexide; direct renin inhibitor: aliskiren; nuclear factor erythroid 2-related factor 2 [NRF-2] activator: bardoxolone methyl; and pentoxyphylline) were also tried in DKD with T2DM, without much success. Indeed, some of these studies showed harm and were stopped prematurely (Avosentan [ASCEND trial], Aliskiren [ALTITUDE trial], VA NEPHRON-D trial, and Bardoxolone [BEACON trial])[13-25].

Nevertheless, after failure of any favorable outcomes for nearly two decades, the year 2019 ushered a new hope for the management of DKD. A series of recent trials have shown a positive renal outcome including a reduction of death in patients with CKD and T2DM, at the top of RASB use. The SONAR (Study of Diabetic Nephropathy with Atrasentan [a selective endothelin A receptor antagonist]), randomized 2648 patients of CKD (eGFR 25-75 mL/min/1.73 m^2 and urinary ACR of 300-5000 mg/g) with T2DM who were receiving a maximum tolerated dose of RASB to either atrasentan 0.75 mg daily or placebo. At a median follow-up of 2.2 years, the primary composite renal endpoint (doubling of serum creatinine or ESKD) was reduced by 35% (HR: 0.65; 95%CI: 0.49-0.88; P = 0.005) in atrasentan *vs* placebo. However, a higher frequency of HHF (33%) and death (9%) was also noted with atrasentan compared to the placebo[26]. Meanwhile, several cardiovascular outcome trials (CVOTs) conducted with SGLT-2 inhibitors (SGLT-2i) in patients with T2DM, with or without DKD (EMPA-REG, CANVAS Program, and DECLARE-TIMI conducted with empagliflozin, canagliflozin, and dapagliflozin, respectively), have also shown a significant reduction in prespecified renal composite endpoints including progression to ESKD, albeit the renal outcomes were exploratory in nature in all these studies [27-29]. Similarly, studies conducted with non-selective steroidal mineralocorticoid receptor antagonists (MRAs) such as spironolactone and eplerenone have shown a significant reduction in proteinuria in patients with CKD although no conclusive evidence is yet available suggesting that these drugs prevent the progression of DKD. While a meta-analysis of 16 RCTs conducted with spironolactone in CKD at the top of RASB showed a significant reduction in proteinuria (although at the increased risk of hyperkalemia[30], a recent (2020) proteomic prediction and renin-angiotensin-aldosterone system inhibition prevention of early diabetic nephropathy in type 2 diabetic patients with normoalbuminuria study failed to show prevention of progression to microalbuminuria with spironolactone, at the end of 2.5 years of follow-up[31]. Another recently updated (2020) Cochrane meta-analysis involving 44 trials of steroidal MRA (spironolactone and eplerenone) in early stage-CKD (mild-to-moderate proteinuria) showed a significant reduction in proteinuria but an increased risk of hyperkalemia (2.17-fold), acute kidney injury (2.04-fold) and gynecomastia (5.14-fold) was noted with spironolactone[32]. Moreover, the latest (2021) Cochrane meta-analysis of 16 trials of steroidal MRA in late-stage CKD requiring dialysis showed a significant reduction in CV- and all-cause mortality but with a significant 6-fold increased risk of gynecomastia and 1.4-fold increased trend of hyperkalemia[33]. However, the major limitations of these meta-analyses include smaller numbers, shorter duration of studies, and potential risk of bias. Indeed, one RCT of spironolactone (Mineralocorticoid Receptor Antagonists in End-Stage Renal Disease trial, commonly known as MiREnDa) that assessed the safety and CV outcomes with spironolactone and another RCT (Spironolactone in Dialysis-Dependent ESRD, commonly known as SPin-D)-both failed to show any benefit on the left ventricular mass index (LVMI) over 40 wk, or diastolic function or LVMI over 36-wk, respectively along with a dose-dependent increased risk of hyperkalemia[34,35]. Similarly, an eplerenone pilot trial PHASE (Hemodialysis patients undergoing Aldosterone Antagonism with Eplerenone) failed to show any CV benefit and had a 4.5-fold increased risk of hyperkalemia against placebo[36].

SGLT-2i era

While SGLT-2i indicated improved renal outcomes in CVOTs of empagliflozin, canagliflozin, and dapagliflozin (EMPA-REG, CANVAS Program, and DECLARE-TIMI, respectively), the results of the first dedicated renal outcome study CREDENCE (Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation) in patients with DKD became available in the year 2019. CREDENCE trial randomized 4402 patients with CKD (eGFR 30 to < 90 mL/min/1.75 m^2 and urinary ACR 300-5000 mg/g) and T2DM already receiving RASB, to either canagliflozin 100 mg daily or placebo. At a median follow up of 2.62 years, the relative risk reduction of primary composite outcome (composite of ESKD, a doubling of the serum creatinine level, or death from renal or CV causes) was 30% (HR: 0.70; 95%CI: 0.59-0.82; P = 0.00001) lower with canagliflozin compared to placebo. ESKD reduced by 31% (HR 0.68; 95%CI: 0.54-0.86; P = 0.002) with canagliflozin compared to placebo. The secondary CV outcome, a composite of 3P-MACE (CV death, non-fatal MI and non-fatal stroke) was found to reduce by 20% (HR: 0.80; 95%CI: 0.67-0.95; P = 0.01), while HHF reduced by 39% (HR: 0.61; 95%CI: 0.47-0.80; P < 0.001) with canagliflozin when compared to placebo[37]. The results of the second kidney outcome trial (Dapagliflozin and Prevention of Adverse Outcomes in Chronic Kidney Disease [DAPA-CKD]) was published in the year 2020. DAPA-CKD randomized 4304 patients with CKD (eGFR 25 to 75 mL/min/1.73 m^2 and urinary ACR of 200 to 5000 mg/g) having 2906 patients with T2DM, to either dapagliflozin 10 mg or placebo. Over a median of 2.4 years, the primary outcome (composite of the sustained decline of eGFR of at least 50%, ESKD, or death from renal or cardiovascular cause) was 39% (HR: 0.61; 95%CI: 0.51-0.72; P < 0.001) lower with dapagliflozin compared to placebo. Reduction in primary renal composite was similar in patients both with (HR: 0.64; 95%CI: 0.52-0.79) or without (HR: 0.50; 95%CI: 0.35-0.72) T2DM with dapagliflozin *vs* placebo. The secondary CV endpoints (composite of CV death or HHF) were reduced by 29% (HR: 0.71; 95%CI: 0.55-0.92; P = 0.009), while all-cause death was reduced by 31% (HR: 0.69; 95%CI: 0.53-0.88; P = 0.004) with dapagliflozin compared to placebo[38]. Ongoing empagliflozin renal outcome trial (EMPA-KIDNEY) in patients with CKD due to either T2DM or non-diabetic cause has been recently (March 16, 2022) stopped owing to the positive results which met the prespecified threshold for early termination against placebo[39]. It should be noted however that the residual risk of CKD progression or kidney failure was still evident in CREDENCE and DAPA-CKD in about 10% of patients despite a full dose of concomitant RASB use after a median follow-up of nearly 2.5 years[37,38]. This necessitates further strategies to combat the progression of DKD in patients with T2DM.

MRA era

While several studies of steroidal MRA (spironolactone and eplerenone) have shown a significant reduction in soft surrogates of proteinuria in patients with DKD albeit, at increased risk of hyperkalemia and gynecomastia (spironolactone), no conclusive evidence of benefit is yet available with these MRAs concerning prevention of ESKD progression. Two ongoing phase 3b RCTs of spironolactone are currently evaluating the CV effect in patients with CKD on dialysis. While the ALdosterone Antagonist Chronic HEModialysis Interventional Survival Trial (commonly known as ALCHEMIST; NCT01848639) is evaluating the primary composite endpoint of non-fatal MI, acute coronary syndrome, HHF, nonfatal stroke, or CV death; the Aldosterone bloCkade for Health Improvement EValuation in End-stage Renal Disease (commonly known as ACHIEVE; NCT03020303) trial is evaluating the composite of CV death or HHF, in patients on maintenance dialysis. The results of both studies are expected in 2023[40].

Meanwhile, several newer, selective, non-steroidal MRA such as finerenone, esaxerenone, and apararenone have also been tried in DKD. The Mineralocorticoid Receptor Antagonist Tolerability Study in Diabetic Nephropathy (ARTS-DN) study, which evaluated various doses of finerenone, showed a dose-dependent significant reduction in UACR (24% and 38% reduction with 10 and 20 mg, respectively) in patients with T2DM having albuminuria (UACR ≥ 30 mg/g) and eGFR of > 30 mL/min/1.73 m^2 at the top of RASB use, although no difference in ≥ 30% decline in eGFR (secondary outcome) was noted against placebo[41]. Significant reduction in proteinuria was also exhibited by esaxerenone in the Esaxerenone in Patients with Type 2 Diabetes and Microalbuminuria (ESAX-DN) study and apararenone study in patients with DKD and T2DM[42,43]. Nevertheless, the conclusive evidence to prevent progression of DKD with MRA was first noted only with finerenone in The Finerenone in Reducing Kidney Failure and Disease Progression in DKD (FIDELIO-DKD) trial that become available in the year 2020. FIDELIO-DKD randomized 5734 patients with CKD (eGFR 25 to < 60 mL/min/1.73 m^2, urinary ACR of 30 to < 300 mg/g and diabetic retinopathy, or urinary ACR 300-5000 mg/g and eGFR 25 to < 75 mL/min/1.73 m^2) and T2DM on maximum licensed dose of RASB, to either finerenone 10 mg (< 60 mL/min/1.73 m^2) or 20 mg (≥ 60 mL/min/1.73 m^2) once daily, or placebo. At the median follow-up of 2.6 years, FIDELIO-DKD showed an 18% reduction (HR: 0.82; 95%CI: 0.73-0.93; P = 0.001) in primary renal outcome (composite of kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes) with finerenone compared to placebo. A significant reduction of 14% (HR: 0.86; 95%CI: 0.75-0.99; P = 0.03) in secondary CV outcome (composite of CV death, nonfatal MI, and nonfatal stroke, or HHF) was also shown with finerenone compared to placebo. Although adverse events were similar in both arms, hyperkalemia-related drug discontinuation was 2.5 times higher with finerenone (2.3%) compared to placebo (0.9%)[44]. Another study conducted with

Table 1 Studies (in chronological order) that evaluated hard renal or cardiovascular composites in patients having diabetic kidney disease and type 2 diabetes mellitus with various pharmacological agents

Ref.	*n*	Comparator	Duration (mean/median)	Primary endpoints	Results	Remarks
Lewis *et al* [11], 2001, IDNT	1715	Irbesartan 75-300 mg *vs* Amlodipine 2.5-10 mg *vs* PBO	2.6 yr	Composite of doubling of serum Cr, development of ESKD or death from any cause	The primary outcome with IRBE was 20% lower than PBO and 23% lower than AMLO. Doubling of Cr was significantly 33% lower in IRBE *vs* PBO ($P = 0.003$) and 37% lower with IRBE *vs* AMLO ($P < 0.001$). The risk of ESKD was non-significantly 23% lower *vs* both PBO and AMLO ($P = 0.07$, for both comparisons). No difference in CV- or all-cause death was noted	Similar BP control with IRBE and AMLO. Protection is independent of reduction in BP
Brenner *et al* [12], 2001, RENAAL	1513	Losartan 50-100 mg *vs* PBO	3.4 yr	Composite of doubling of serum Cr, development of ESKD or death from any cause	Primary outcome reduced by 16% risk in LOSA *vs* PBO ($P = 0.020$). LOSA reduced the doubling of Cr by 25% ($P = 0.006$) and ESKD by 28% ($P = 0.002$) *vs* PBO but no effect on death was noted. HHF was reduced by 32% in LOSA ($P = 0.005$) while proteinuria was reduced by 35% ($P < 0.001$) *vs* PBO	There was no active comparator, and the mean blood pressure throughout the study was lower among those assigned losartan
Wanner *et al* [13], 2005, 4D	1255	Atorvastatin 20 mg *vs* PBO (receiving hemodialysis)	4.0 yr	Composite of 3P-MACE (death from CV causes, nonfatal MI, and stroke)	No benefit in 3P-MACE (RR: 0.92; 95%CI: 0.77-1.10; $P = 0.37$) but significant increase in fatal stroke (RR: 2.03; $P = 0.04$)	An increase in stroke could be a chance finding, given the data from the CARDS trial that showed atorvastatin lowers the incidence of stroke (HR: 0.52; 95%CI: 0.31-0.89)
Tuttle *et al* [14], 2007, PKC-DRS, PKC-DMES and PKC-DRS 2 study	1157	Ruboxistaurin *vs* PBO	33-39 mo	Composite of doubling of serum Cr, development of advanced chronic kidney disease (stages 4 to 5), and death	No difference between the two group	-
Pfeffer *et al* [15], 2009, TREAT	4038	Darbepoetin alfa *vs* PBO	4.0 yr	Composite outcomes of death or a CV event (nonfatal MI, CHF, stroke, or hospitalization for myocardial ischemia) and of death or ESKD	No difference in the composite of death or ESKD (HR: 1.06; 95%CI: 0.95-1.19; $P = 0.29$) or ESKD (HR: 1.02; 95%CI: 0.87-1.18; $P = 0.83$) between darbepoetin alfa and PBO. An increase in stroke (fatal or nonfatal stroke) occurred in darbepoetin alfa compared with PBO (HR: 1.92; 95%CI: 1.38-2.68; $P < 0.001$)	-
Mann *et al*[16], 2010, ASCEND	1392	Avosentan 25/50 mg *vs* PBO	4 mo	Composite of doubling of serum Cr, ESKD, or death	No difference in primary outcome (25 mg 8.1% *vs* PBO 9.6%; $P = 0.46$; 50 mg 8.6% *vs* PBO 9.6%; $P = 0.79$) but a significant increase in CHF with avosentan (25 mg 5.9% *vs* PBO 2.2%; $P = 0.008$; 50 mg 6.1% *vs* PBO 2.2%; $P = 0.05$) compared with PBO	The trial terminated prematurely after a median follow-up of 4 mo (maximum 16 mo) because of an excess of CV events with avosentan
Sharma *et al* [17], 2011	77	Pirfenidone 1200/2400 mg *vs* PBO	1 yr	Change in eGFR	Mean eGFR significantly increased the pirfenidone 1200-mg/d group *vs* PBO (+3.3 *vs* -2.2 mL/min; $P = 0.03$) but no improvement in 2400-mg/d group	-
Pergola *et al* [18], 2011, BEAM	227	Bardoxolone 25/75/150 mg OD *vs* PBO	12 mo	Change in eGFR at 6 mo	Significant increase in mean eGFR both at 6-mo (8.2-11.4 mL/min; $P < 0.001$) and 12-mo (5.8-10.5 mL/min) against PBO	Muscle spasms were the MC observed S/E with BDX
Lewis *et al* [19], 2012	317	Pyridoxamine 150/300 mg BID *vs* PBO	52-wk	Change in serum Cr	No difference in outcome observed	-

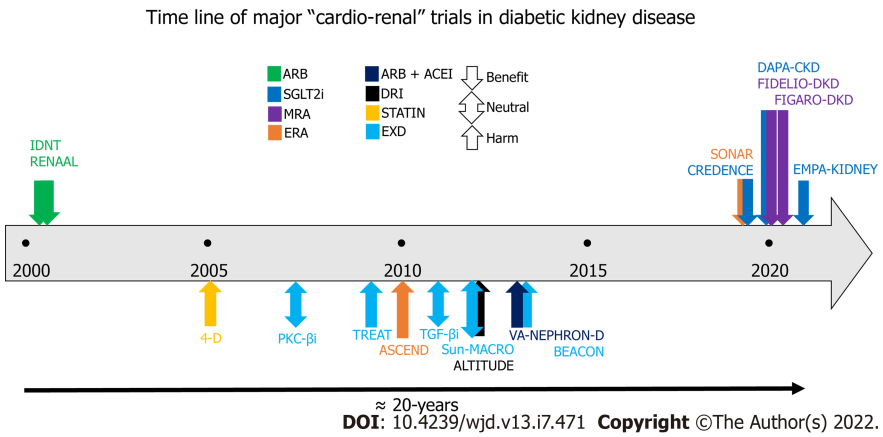
Packham *et al* [20], 2012, Sun-MACRO	1248	Sulodexide *vs* PBO	11 mo	Composite of a doubling of serum Cr, development of ESKD, or serum Cr ≥6.0 mg/dl	No difference in the outcome	The trial was stopped prematurely due to futility
Parving *et al* [21], 2012, ALTITUDE	8561	Aliskiren *vs* PBO	32.9 mo	Composite of CV death or the first occurrence of cardiac arrest with resuscitation; nonfatal MI; nonfatal stroke; unplanned HHF; ESKD, death attributable to kidney failure, or the need for RRT with no dialysis or transplantation available or initiated; or doubling of Cr level	Results of the primary endpoint were no different between the two arms (HR: 1.08; 95% 0.98-1.20; $P = 0.12$)	The trial was stopped prematurely after the second interim efficacy analysis because of significantly higher (11.2% *vs* 7.2%; $P < 0.001$) hyperkalemia (Serum K level ≥ 6 mmol/L) and hypotension (12.1% *vs* 8.3%; $P < 0.001$ in the aliskiren group *vs* PBO
Fried *et al*[22], 2013, VA NEPHRON-D	1448	Losartan plus lisinopril *vs* losartan plus PBO	2.2 yr	Composite of change in the eGFR, ESKD, or death	No difference in outcome (HR: 0.88; 95%CI: 0.70 to 1.12; $P = 0.30$). Combination therapy increased the risk of hyperkalemia ($P < 0.001$) and acute kidney injury ($P < 0.001$) compared to monotherapy	The trial was stopped prematurely
Mann *et al*[23], 2013, ONTARGET	3163	Ramipril 10 mg *vs* telmisartan 80 mg *vs* ramipril plus telmisartan (10 + 80)	56-mo	Composite of dialysis, doubling of serum Cr, and death	Combination therapy was associated with a non-significantly higher ESKD or doubling of serum creatinine (5.3% *vs* 4.8 %), but a similar death rate (2.3% *vs* 2.2 %) *vs* monotherapy. Combination therapy had higher rates of acute kidney injury requiring dialysis (1.4% *vs* 0.8 %)	This is the data of 3163 people having DKD from a total of 9628 patients with diabetes
de Zeeuw *et al* [24], 2013, BEACON	2185	Bardoxolone 20 mg OD *vs* PBO	9.0 mo	Composite of ESKD or CV death	No difference (HR: 0.98; 95%CI: 0.70-1.37; $P = 0.92$). Significant increase in HHF and death due to HHF with bardoxolone (HR: 1.83; 95%CI: 1.32-2.55; $P < 0.001$) *vs* PBO	The trial was stopped prematurely
Navarro-Gonzalez *et al* [25], 2015, PREDIAN	169	Pentoxyphylline 600 mg BID *vs* PBO	2-yr	Change in eGFR	Significant less decrease in eGFR in PTF *vs* PBO (-2.1 *vs* -6.5 mL/min; Group difference -4.3 mL/min; $P < 0.001$)	Open-label design and envelope (rather than computer-generated) randomization could have biased the results
Heerspink *et al* [26], 2019, SONAR	2648	Atrasentan 0.75 mg *vs* PBO	2.2 yr (Median)	Composite of doubling of serum Cr or ESKD or death from kidney failure	35% reduction in primary composite renal endpoint event (HR: 0.65; 95%CI: 0.49-0.88; $P = 0.005$)	HHF was insignificantly higher in atrasentan (HR: 1.33; 95%CI: 0.85-2.07; $P = 0.208$) *vs* PBO
Perkovic *et al* [37], 2019, CREDENCE	4401	Canagliflozin 100 mg *vs* PBO	2.6 yr	Composite of ESKD, doubling of serum Cr, or death from renal or CV causes	30% reduction in primary composite (HR: 0.70; 95%CI: 0.59-82; $P = 0.00001$), 34% reduction (HR: 0.66; 95%CI: 0.53-0.81; $P < 0.001$) in renal-specific composite (of ESKD, doubling of Cr, renal death) and 32% reduction in ESKD (HR: 0.68; 95%CI: 0.54-0.86; $P = 0.002$) in CANA *vs* PBO. Reduction in composite of 3P-MACE was 20% (HR: 0.80; 95%CI: 0.67-0.95; $P = 0.01$) while HHF reduced by 39% (HR: 0.61; 95%CI: 0.47-0.80; $P < 0.001$) in CANA arm *vs* PBO	The trial was stopped prematurely due to efficacy
Heerspink *et al* [38], 2020, DAPA-CKD	4304	Dapagliflozin 10 mg *vs* PBO	2.4 yr	Composite of ESKD, sustained decline in eGFR of at least 50%, or death from renal or CV causes	39% reduction in primary composite (HR: 0.61; 95%CI: 0.51-0.72; $P < 0.001$), 44% reduction (HR: 0.56; 95%CI: 0.45-0.68; $P < 0.001$) in renal-specific composite (of ESKD, decline in eGFR of at least 50%, or renal death), 29% reduction (HR: 0.71; 95%CI: 0.55-0.92; $P = 0.009$) in composite of CV death of HHF and 31% reduction in death (HR: 0.69;	The trial stopped prematurely due to efficacy

					95%CI: 0.53-0.88; *P* = 0.004) in DAPA arm *vs* PBO	
Bakris *et al* [44], 2020, FIDELIO-DKD	5734	Finerenone 10/20 mg *vs* PBO	2.6 yr	Composite of kidney failure, sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes	18% reduction (HR: 0.82; 95%CI: 0.73-0.93; *P* = 0.001) in primary composite in FINE *vs* PBO arm. 14% reduction (HR: 0.86; 95%CI: 0.75-0.99; *P* = 0.03) in secondary outcome composite (CV death, non-fatal MI, non-fatal stroke, or HHF) in FINE *vs* PBO arm	Hyperkalemia-related discontinuation of the drug was higher in the FINE *vs* PBO (2.3% *vs* 0.9%) arm
Pitt *et al*[45], 2021, *FIGARO*-DKD	7437	Finerenone 10/20 mg *vs* PBO	3.4 yr	Composite of CV death, nonfatal MI, nonfatal stroke, or HHF. The secondary outcome was a composite of a decrease of eGFR by at least 40%, ESKD, or death from renal causes	13% reduction (HR: 0.87; 95%CI: 0.76-0.98; *P* = 0.03) in primary cardiac composite primarily driven due to 29% reduction (HR: 0.71; 95%CI: 0.56-0.90) in HHF with FINE *vs* PBO. Non-significant 13% reduction (HR: 0.87; 95%CI: 0.76-1.01) in secondary renal composite with FINE *vs* PBO	Hyperkalemia-related discontinuation of the drug was higher in the FINE *vs* PBO (1.2% *vs* 0.4%) arm

3P-MACE: 3-point major adverse cardiac events; AMLO: Amlodipine; BDX: Bardoxolone; BID: Twice daily; BP: Blood pressure; CANA; Canagliflozin; CHF: Congestive heart failure; CI: Confidence interval; Cr: Creatinine; CV: Cardiovascular; DAPA; Dapagliflozin; DKD: Diabetic kidney disease; eGFR: Estimated glomerular filtration rate; ESKD: End-stage kidney disease; FINE: Finerenone; HHF: Heart failure hospitalization; HR; Hazard ratio; IRBE: Irbesartan; LOSA: Losartan; MC: Most common; MI: Myocardial infarction; OD: Once daily; PBO: Placebo; PTF; Pentoxyphylline; RR: Relative risk; RRT: Renal replacement therapy; S/E: Side effects.

Finerenone FIGARO-DKD (Reducing Cardiovascular Mortality and Morbidity in DKD) has been published recently in 2021. FIGARO-DKD trial randomized 7437 patients with CKD (eGFR 25 to 90 mL/min/1.73 m^2 and urinary ACR of 30 to < 300 mg/g, or urinary ACR 300 to 500 mg/g and eGFR ≥ 60 mL/min/1.73 m^2) and T2DM to either finerenone 10 mg (25 to < 60 mL/min/1.73 m^2) or 20 mg (≥ 60 mL/min/1.73 m^2) once daily, or placebo on the maximum licensed dose of RASB. On a median follow-up of 3.4 years, the primary CV outcome (composite of CV death, nonfatal MI, and nonfatal stroke, or HHF) was significantly reduced by 13% (HR: 0.87; 95%CI: 0.76-0.98; *P* = 0.03) primarily driven by 29% reduction (HR: 0.71; 95%CI: 0.56-0.90) in HHF with finerenone compared to placebo. Interestingly, no significant difference (HR: 0.87; 95%CI: 0.76-1.01) was noted in secondary renal outcome (composite of kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal cause) with finerenone compared to placebo. Overall, no difference in the adverse events was noted in the two arms, however hyperkalemia-related drug discontinuation was 3-times higher with finerenone (1.2%) compared to placebo (0.4%)[45]. Table 1 summarizes the results from all these studies (in chronological order) which have been conducted in patients with T2DM having CKD that evaluated hard renal or cardiovascular composite endpoint as the primary objective[11-26,37,38,44,45]. Figure 1 is a schematic representation of timelines and outcomes from all these cardio-renal outcome trials.

In summary, several agents have been tried in the past two decades in patients with DKD and T2DM, but only three drug classes (RASB, SGLT-2i, and MRA especially finerenone) have conclusively shown both ≥ 30% reduction in albuminuria and a significant lowering in renal disease progression. It should be recalled that a cut-off of 30% geometric mean albuminuria reduction within 6 mo or an eGFR slope reduction of 0.5-1.0 mL/min/1.73 m^2/year over 2-3 years has been adopted as a surrogate renal endpoint for CKD progression for clinical trials by National Kidney Foundation, European Medicines Agency, and US Food and Drug Administration in the year 2020[46]. This cut-off seems to have primarily originated from at least two meta-analyses[47,48]. While the Reducing Albuminuria as Surrogate Endpoint (REASSURE) Consortium showed each 30% reduction in albuminuria lowered the risk of ESKD by 24%, a meta-analysis of observational studies involving nearly 700000 individuals found that a 30% reduction of albuminuria over 2 years lowered ESKD by 22%, regardless of drug class tested[47,48]. However, the pressing question which remains unanswered conclusively is whether the addition of MRA including finerenone to the patients who are already receiving SGLT-2i and RASB would help prevent further progression of kidney disease[49]. Mechanistically, the action of both SGLT-2i and MRA including finerenone appears to be complementary due to the following: (1) The differential mechanism of action. While SGLT-2i reduces glomerular hyperfiltration and could have direct beneficial cellular and metabolic effect, finerenone reduces inflammation and fibrosis by inhibiting mineralocorticoid receptor pathway; and (2) Hyperkalemia induced by finerenone (the commonest reason for drug discontinuation) can be counterbalanced by SGLT-2i. A recent meta-analysis from the pooled data of five RCTs (*n* = 8296) in patients with reduced ejection fraction showed SGLT-2i plus MRA to significantly reduce both cardiovascular composite of CV death or HHF (HR: 0.73; 95%CI: 0.66-0.80; *P* < 0.00001) and composite renal endpoints (HR 0.56; 95%CI: 0.39-0.81; *P* = 0.002) but with a significantly lower risk of hyperkalemia (HR 0.60; 95%CI: 0.42-0.87; *P* = 0.007), compared to MRA alone [50]. However, renal outcomes were exploratory endpoints in these RCTs included in this meta-analysis.



DOI: 10.4239/wjd.v13.i7.471

Figure 1 Major cardio-renal outcome trials in patients with diabetic kidney disease and type 2 diabetes mellitus. ARB: Angiotensin-receptor blocker; ACEI: Angiotensin converting enzyme inhibitors; DRI: Direct renin inhibitors; ERA: Endothelin A receptor antagonists; EXD: Experimental drugs; MRA: Mineralocorticoid receptor antagonists; PKC-βi: Protein-kinase C β inhibitor; SGLT-2i: Sodium-glucose co-transporter 2 inhibitors; TGF-βi: Tumor growth factor β inhibitor.

In FIDELIO-DKD, 4.6% (259/5674) patients were receiving SGLT-2i at the baseline and reduction in primary renal composite was similar ($P_{\text{Interaction}}$ = 0.21), regardless of the SGLT-2i use (SGLT-2i users: HR, 1.38; 95%CI: 0.61-3.10; SGLT-2i non-users: HR, 0.82; 95%CI: 0.72-0.92). Similarly, in FIGARO-DKD, 8.4% patients (618/7352) were receiving SGLT-2i at baseline and benefit in primary CV composite was similar, regardless of SGLT-2i use (SGLT-2i users: HR, 0.49; 95%CI: 0.28-0.86; SGLT-2i non-users: HR, 0.89; 95%CI: 0.78-1.01). Importantly, a recent subgroup analysis of FIDELIO-DKD found that finerenone caused a 25% reduction in UACR in patients receiving SGLT-2i at the baseline, and patients on SGLT-2i also had fewer hyperkalemia events. Indeed, this subgroup analysis stratified on the baseline SGLT-2i use reported a lesser episode of treatment-emergent hyperkalemia of both moderate (> 5.5 mmol/L) and severe (> 6.0 mmol/L) nature in combined SGLT-2i plus finerenone users (7% and 0%, respectively), compared with finerenone alone (22% and 5%, respectively)[51]. Notably, a recent meta-analysis of six cardio-renal trials involving 49875 individuals has found a 16% lower risk (HR: 0.84; 95%CI: 0.76-0.93) of serious hyperkalemia (> 6.0 mmol/L) with SGLT-2i without any higher risk of hypokalemia[52]. Collectively, these finding hints that combination therapy of SGLT-2i and finerenone would likely reduce the risk of hyperkalemia. Whether combining MRA to SGLT-2i would enhance the CV or renal outcome is not clearly known due to: (1) Low number of events in a small population of baseline SGLT-2i users in both FIDELIO-DKD and FIGARO-DKD trial (number of events 24 and 61, respectively); and (2) Absence of any dedicated RCT that has assessed the renal or CV outcome with the combination therapy in patients with CKD and T2DM. Efficacy and safety of finerenone plus empagliflozin compared with either finerenone or empagliflozin in 807 participants with CKD and T2DM (CONFIDENCE Trial, NCT05254002) is currently planned and expected to be complete by end of 2023[53].

CONCLUSION

While optimal glucose control, intensive blood pressure control, and use of RASB have been the traditional foundation of treatment in slowing the progression of kidney disease in patients with albuminuria and T2DM for the past two decades, the addition of SGLT-2i to this foundational treatment has further shown to reduce the disease progression including death (DAPA-CKD). Finerenone would be a welcome addition to the list of novel drugs that have been able to reduce the progression of CKD successfully in patients with T2DM along with RASB. It is also possible that finerenone plus SGLT-2i combination can further prevent the progression of DKD in T2DM but that has to be proved through dedicated RCTs.

FOOTNOTES

Author contributions: Singh AK designed the research; Singh R performed the research, Singh AK and Singh R analyzed the data; Singh AK wrote the editorial; Singh R revised the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Chang KL
L-Editor: Filipodia
P-Editor: Chang KL

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World Journal of Diabetes

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World J Diabetes 2022 July 15; 13(7): 482-497

DOI: 10.4239/wjd.v13.i7.482

ISSN 1948-9358 (online)

REVIEW

Fetal programming of obesity and type 2 diabetes

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Islam SMRU, Bangladesh; Shuang WB, China; Ulasoglu C, Turkey
A-Editor: Guo L, China

Received: July 9, 2021
Peer-review started: July 9, 2021
First decision: August 8, 2021
Revised: August 18, 2021
Accepted: June 3, 2022
Article in press: June 3, 2022
Published online: July 15, 2022

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Abstract

The prevalence of obesity and type 2 diabetes mellitus has increased rapidly over the past few decades, and prevention efforts have not been successful. Fetal programming involves the earliest stage of obesity development, and provides a novel concept to complement other strategies for lifelong prevention of obesity and type 2 diabetes mellitus. The World Health Organization now advocates a life-course approach to prevent/control obesity, starting with pre-conceptional and antenatal maternal health. Maternal overnutrition, gestational diabetes mellitus and excessive gestational weight gain lead to fetal overgrowth, and "programs" the offspring with an increased risk of obesity and type 2 diabetes mellitus in childhood and adulthood. This review summarizes current data on fetal programming of obesity and type 2 diabetes mellitus including potential causative factors, mechanisms and interventions to reduce its impact.

Key Words: Developmental programming; Metabolic syndrome; Intergenerational obesity cycle; Insulin resistance; *In utero* environment

Core Tip: Fetal programming targets the earliest stages in the development of obesity and type 2 diabetes. It provides a novel paradigm to complement other strategies for lifelong prevention of obesity and type 2 diabetes. Maternal undernutrition/overnutrition, maternal diabetes, excessive gestational weight gain and certain paternal factors are now recognized as factors associated with adverse fetal programming of obesity and type 2 diabetes in the offspring. This review provides up-to date evidence on fetal programming of obesity and type 2 diabetes including potential causative factors and mechanisms as well as potential interventions to minimize its impact on future generations.

Citation: Seneviratne SN, Rajindrajith S. Fetal programming of obesity and type 2 diabetes. *World J Diabetes* 2022; 13(7): 482-497
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/482.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.482

INTRODUCTION

Obesity and type 2 diabetes mellitus rates are rising globally. Obesity is the commonest form of malnutrition in the developed world, and is rapidly increasing in developing countries[1-3]. Obesity is strongly associated with insulin resistance and the development of type 2 diabetes. By 2050, it is predicted that half a billion men, women, and children will have type 2 diabetes, of whom three quarters will be from low and middle income countries (LMIC)[4]. Diabetes and its complications including kidney disease, heart disease, stroke, retinopathy and neuropathy, lead to premature mortality, morbidity, disability and reduced quality of life in affected individuals. At present, someone dies due to diabetes-related complications every 7 s[5]. Furthermore, it also leads to decreased work-force productivity, increased healthcare utilization and escalating healthcare costs[6]. Ten percent of the global health expenditure is spent on diabetes-related care[4].

Obesity is the main driver of type 2 diabetes. Obesity refers to the excess accumulation of body fat to an extent that it is harmful to an individual's health. The fundamental cause of obesity is an imbalance between energy intake and expenditure, with excess energy being stored as fat in adipose tissue. This predisposes adipocytes to secrete more pro-inflammatory adipocytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), causing a state of low-grade inflammation and insulin resistance. An increase in insulin resistance necessitates a compensatory increase in insulin secretion from pancreatic β cells, and failure to achieve this demand results in diabetes. This, however, is a gradual process, and it may take years for diabetes to manifest clinically. Thus, early identification and modification of risk factors at the onset of the above trajectory could help prevent type 2 diabetes[5].

The etiology of obesity and type 2 diabetes is multifactorial and involves complex interactions between genetic, environmental and behavioral factors[3,7]. The rapid rise in obesity is mostly attributed to the unhealthy lifestyle associated with urbanization and technical advancement over the last three to four decades[8]. The present generations live within an obesogenic environment, with energy imbalance arising from excessive energy intake due to high fat, high-sugar, energy-dense processed foods, and a reduction in occupational, household and leisure-time physical activity[3,7,9]. However, there is evidence of an additional factor leading to increases in obesity and type 2 diabetes. This is the impact of the prenatal and early-life environment on long-term health *via* fetal programming.

The Developmental Origins of Health and Disease (DOHaD) concept states that early-life environmental influences at sensitive periods of development lead to lifelong effects on health and chronic disease risk[10]. There is evidence that exposure to an abnormal *in utero* environment disturbs the metabolic programming of the growing fetus, increasing the lifelong risk of chronic diseases including type 2 diabetes[11-15] This process is described as fetal or developmental programming[16]. Fetal programming is now recognized as a key factor contributing to the rapid rise in obesity and type 2 diabetes mellitus rates worldwide. Research in humans and animals over the past two decades has provided considerable evidence supporting 'developmental programming' by the intrauterine environment[17].

Fetal programming helps explain certain aspects of the obesity epidemic that cannot be fully explained by genetic and environmental factors. The relatively short time over which obesity and type 2 rates have escalated precludes genetic change as a major attributor[15,18]. Furthermore, energy homeostasis and body weight are regulated by biological systems established in early life. Thus, it is difficult to explain how lifestyle changes alone, can override these biological homeostatic mechanisms to bring about obesity[15,18]. Fetal programming is the most plausible reason for this phenomenon. Dysregulation of biological mechanisms maintaining body weight by early life fetal programming also helps explain why reversal of obesity is difficult[19].

FETAL PROGRAMMING OF OBESITY AND TYPE 2 DIABETES

Epidemiological, clinical, and basic sciences research suggest that the foundations of an individual's lifelong health, including predisposition towards obesity and type 2 diabetes is largely established during the 'first 1000 days of life' from day of conception to completion of the 2nd year of life. This is a highly sensitive period of growth and development in humans, where biological systems are formed and developed[10,20,21].

It is difficult to separate out effects of *in utero* exposure from genetic and nurturing influences in humans. However, studies in small mammals and other animal models have shown that prenatal

exposure to an adverse *in utero* environment associated with maternal overnutrition results in developmental programming of obesity and other disorders in offspring[22-25]. For example, in genetically-modified obesity-prone rats, greater postnatal adiposity was observed in offspring born to over-nourished dams, compared to normally nourished dams[22]. Furthermore, offspring of over-nourished dams developed greater body weight and body fat compared to offspring of lean dams, even when both groups were fostered by lean dams after birth[23]. These studies indicate that *in utero* exposure to maternal obesity *per se* increases susceptibility to obesity in later life, beyond genetics or nurturing practices.

The prenatal environment in humans appears to be influenced by maternal body composition, metabolism, stress and diet from conception and throughout pregnancy. Paternal influences are also being recognized. Thus, parental lifestyle appears to influence the health of the offspring prior to birth, *via* fetal programming. From the maturation of gametes through to early embryonic development, parental lifestyle can adversely influence long-term risks of offspring metabolic, cardiovascular, immune, and neurological morbidities[26].

FACTORS PREDISPOSING TO DEVELOPMENTAL PROGRAMMING OF OBESITY/TYPE 2 DIABETES AND POTENTIAL MECHANISTIC PATHWAYS

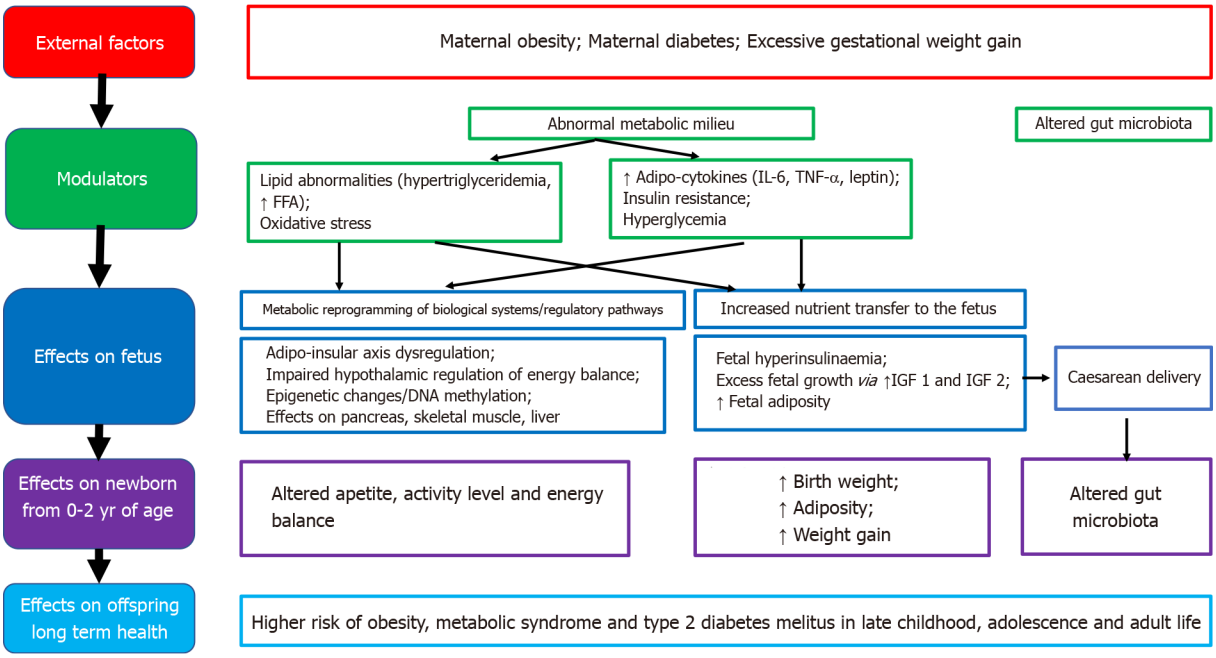
The field of developmental programming has begun to move beyond associations to potential causal mechanisms for developmental programming. Studies in humans and animal models are helping unravel underlying biological mechanisms underpinning fetal programming, including epigenetic, cellular, physiological, and metabolic processes[26]. We have however, still not gained a complete understanding of the complex ways in which the maternal genome, metabolome, and microbiome relate throughout pregnancy and lactation to increase the offspring's disease risk across the life span[25]. Determining mechanisms of fetal programming has been complicated by rapid changes in the social environment and human behavior. Thus, more studies are needed to help better delineate the pathophysiological mechanisms underpinning fetal programming[25].

Epigenetics, and mechanisms of epigenetic modification have led to increased understanding of developmental programming, and how environmental, genetic and epigenetic factors inter-relate to cause lasting effects on offspring size, adiposity and future metabolic outcomes. Neonatal methylation markers associated with birth weight from several gene loci, have shown significant associations with the prenatal environment, as well as longitudinal associations with offspring size and/or adiposity in early childhood, providing evidence that developmental pathways to adiposity begin before birth and are influenced by environmental, genetic and epigenetic factors[16]. Disruption of the gut microbiome observed in maternal obesity, antibiotic use in pregnancy, delivery and early infancy, and cesarean section have also been implicated in increased childhood obesity risk. Disruption of microbiome colonization during critical periods of early development can predispose offspring to obesity, asthma, allergy and diabetes. This may occur due to cesarean delivery, and the use of prophylactic antibiotics during cesarean section, as well as maternal exposure to antibiotics in the second and third trimesters of pregnancy, and use of antibiotics in the offspring in early infancy. Furthermore, increased maternal body mass index (BMI) *per se* is associated with altered intestinal microbial community structure of infants' stool up to 2 years of age[25]. Future research in epigenetics and the gut microbiome could yield greater insights into the mechanistic pathways as well as potential methods of modulating fetal programming.

Good maternal nutrition prior to and during pregnancy is important for optimizing offspring long-term health. Fetal programming, initially described in relation to fetal undernutrition, was associated with a higher risk of central obesity, diabetes, hypertension, coronary heart disease and stroke in adult life[12]. Fetal growth is influenced by the *in utero* environment, and there is trouble at both ends of the birthweight spectrum, with a 'J' shaped relationship between birth weight and future obesity risk[27]. It is proposed that the fetus 'senses' its future nutritional status *via in utero* signals from the mother, and responds in ways which establish lasting influences on weight and appetite control[28]. Many umbilical cord blood metabolites and hormones are associated with birth weight and adiposity in human infants [25]. Paradoxically, both a nutritionally limited or nutritionally excessive *in utero* environment can lead to later obesity and associated co-morbidities[29]. More recent evidence emphasizes the adverse developmental programming effects of fetal overnutrition, and its association with increased risk of obesity in childhood and adulthood[29,30]. In addition, paternal factors are also now being recognized to play a role in fetal programming. The effects of maternal overnutrition, maternal undernutrition/stress and paternal factors on fetal programming of obesity/type 2 diabetes including potential modulatory pathways and effects on the offspring are shown in Figures 1-3.

Maternal overnutrition

Concurrent with the global epidemic of obesity, the prevalence of overweight and obesity in women of reproductive age has risen rapidly over the last three decades[7,31,32]. There is now compelling evidence from human as well as animal studies that maternal obesity, diabetes and increased gestational



DOI: 10.4239/wjd.v13.i7.482

Figure 1 Associations between maternal overnutrition and fetal programming of obesity/type 2 diabetes mellitus including potential modulating factors and effects on offspring health. FFA: Free fatty acid; TNF-α: Tumor necrosis factor alpha; IL-6: Interleukin-6; IGF: Insulin-like growth factor.

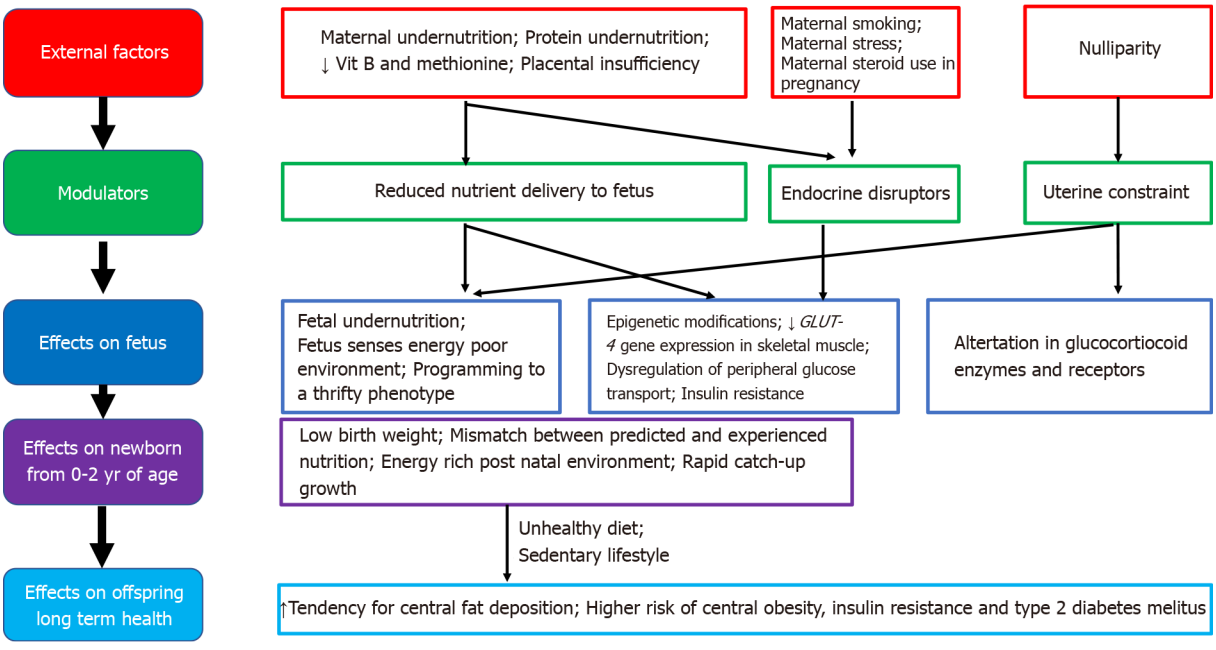


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Figure 2 Associations between maternal undernutrition and fetal programming of obesity/type 2 diabetes mellitus including potential modulating factors and effects on offspring health.

weight gain all increase offspring birth weight and lead to fetal programming of obesity in the offspring [33]. It is thought that offspring obesity is programmed by the 'obesogenic' maternal metabolic environment the fetus is exposed to *in utero* during development, setting in an 'obesity cycle', where maternal obesity leads to neonatal obesity which continues to childhood and adulthood, propagating obesity in the next generation[34,35]. Thus, an increase in overweight and obesity among women of reproductive age should be considered an important modulator of the global obesity epidemic, which is further propagating obesity in future generations.

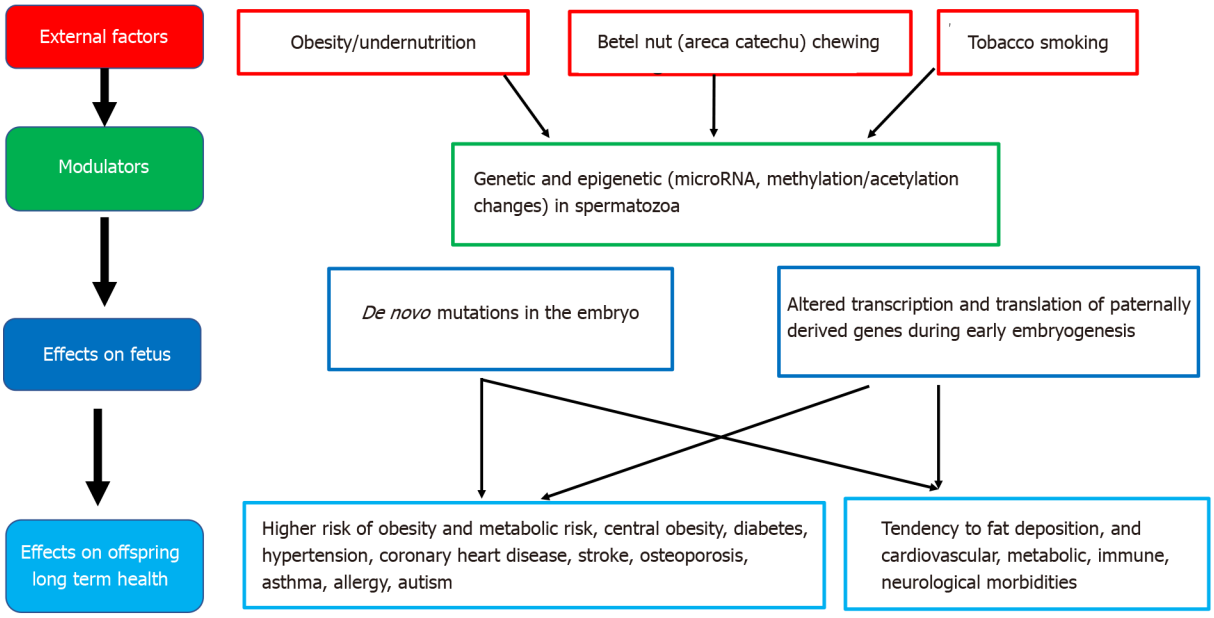

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Figure 3 Associations between paternal health factors and fetal programming of obesity/type 2 diabetes mellitus including potential modulating factors and effects on offspring health.

Children born to overweight/obese women have increased birth weight and an increased risk of obesity and metabolic dysregulation throughout life[34,36-38]. At birth, these babies have increased birth weight and adiposity[39], and thus, an increased risk of assisted delivery as well[40,41]. Exposure to maternal obesity and diabetes accelerates adipogenesis and impairs energy sensing, affecting neurodevelopment, liver, pancreas, and skeletal muscle development in the offspring, creating a lifelong impact on multiple systems[25]. The influence of maternal obesity on the risk of offspring obesity starts manifesting from early life[36,42]. These children show increased weight for age and length, in comparison to offspring of normal weight women, as early as six months of age[42], and their risk of obesity is increased two-fold as preschoolers, even after controlling for birth weight and other confounding factors[36]. They also have an increased risk of metabolic syndrome by late childhood[43]. Furthermore, high maternal BMI in pregnancy is an independent predictor of obesity in the adult offspring, at 30 years of age[37].

If the mother is obese during pregnancy, there is excess transfer of nutrients to the fetus, stimulating increased fetal insulin secretion, fetal overgrowth and increased adiposity. It is hypothesized that this tendency for fat accrual then tends to persist during childhood and adulthood. Furthermore, the metabolic milieu of overweight/obese mothers differs from normal weight mothers, with obese pregnancy being associated with higher insulin resistance, pro-inflammatory adipokines (leptin, IL-6, TNF-α) and lipid abnormalities. *In utero* exposure to this abnormal metabolic milieu is also implicated in fetal programming[31,35,44]. Factors associated with developmental programming of obesity in offspring of mothers who have obesity/diabetes in pregnancy include high glucose levels, triglycerides, free fatty acids, adiponectin, leptin, hypoxia, oxidative stress, inflammation, and the microbiome[25]. It is proposed that fetal exposure to this abnormal metabolic milieu leads to dysregulation of the offspring adipo-insular axis (leptin and insulin) causing alterations in the central nervous system regulation of appetite, activity level, energy balance and in adipocyte metabolism[14,34,44,45].

Maternal diabetes

Maternal diabetes during pregnancy is also strongly associated with fetal programming of obesity in the offspring. At present, approximately 20 million live births are affected by hyperglycemia in pregnancy, globally[4]. There is evidence that offspring of mothers with gestational diabetes mellitus have an increased risk of developing obesity, insulin resistance, type 2 diabetes, hypertension and cardiovascular complications at a relatively young age[46]. In the follow-up of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study, 4160 children aged between 10-14 years, whose mothers had a 75-g oral glucose tolerance test at 28 wk of gestation demonstrated that exposure to higher maternal glucose spectrum levels *in utero* was significantly associated with childhood glucose levels and insulin resistance, independent of maternal and childhood BMI and family history of diabetes[47]. Studies have also shown associations between the sex of the fetus and maternal blood glucose concentrations during pregnancy, suggesting that fetal programming could be influenced by offspring gender as well[48,49].

Maternal undernutrition

Maternal undernutrition during critical periods of fetal development has been linked with fetal programming of central obesity, insulin resistance, metabolic syndrome and type 2 diabetes in later life, especially when exposed to an energy-rich diet postnatally. Adipogenesis, which begins *in utero* and accelerates in neonatal life, is a major candidate for developmental programming. According to the thrifty phenotype hypothesis, maternal undernutrition during critical periods leads to compensatory changes in the fetus, including tendency to store fat, which causes central obesity in later life, when there is a mismatch between the predicted and experienced postnatal nutritional environment[50,51].

Epigenetic pathways in fetal programming from *in utero* undernutrition described include histone modifications in skeletal muscle that directly decrease *GLUT-4* gene expression, which leads to metabolic dysregulation of peripheral glucose transport and insulin resistance, which can contribute to the development of type 2 diabetes in later life. These fetal programming changes, combined with the effects of obesity, ageing and physical inactivity, are the most important factors in determining type 2 diabetes in those born with low birthweight[51]. Furthermore, specific maternal nutrient deficiencies during pregnancy, including low maternal protein consumption, and poor vitamin B and methionine status are also associated with an increased risk of metabolic derangements and type 2 diabetes in later life. Evidence from animal studies show that a protein-restricted diet *in utero* programs susceptibility to obesity, when exposed to overnutrition in postnatal life[50,52].

Prenatal stress could also be a modulating factor for fetal programming of obesity in severe maternal malnutrition[53]. During fetal development, the hypothalamic-pituitary-adrenal axis is extremely susceptible to programming, and alterations in the expression and function of glucocorticoid receptors and major glucocorticoid regulatory enzymes are observed in those exposed to undernourishment in early life[54]. Other factors associated with fetal programming include maternal exposure to endocrine disruptors, maternal infection and smoking and nulliparity[17,55]. Nulliparity is potentially associated with subtle adverse metabolic outcomes in overweight/obese mothers and their offspring, through uterine constraint effects[55].

Paternal factors

Epidemiological and animal studies suggest that many factors, including paternal under- and over-nutrition, exposure to environmental toxins, father's health conditions such as diabetes, and even grandfather's nutritional status can program diseases in the offspring *via* germ cell-mediated transmission[56]. High paternal BMI has been linked with newborn adiposity[57]. Furthermore, paternal overweight/obesity appears to induces paternal programming of offspring phenotypes, through genetic and epigenetic changes in spermatozoa. Both human and rodent models have established that paternal obesity impairs sex hormones, basic sperm function, and molecular composition, which can result in perturbed embryo development and increase subsequent offspring disease burden[57]. Theories for the origin of male obesity-induced paternal programming include the accumulation of sperm DNA damage resulting in de novo mutations in the embryo and changes in sperm epigenetic marks (microRNA, methylation, or acetylation) altering the access, transcription, and translation of paternally derived genes during early embryogenesis[57].

Postnatal factors

In keeping with the concept of "the first 1000 days of life", postnatal factors from the time of birth to the second birthday of a child, could also contribute towards adverse programming increasing the risk of obesity and type 2 diabetes in later life. Our present state of knowledge includes mainly early life nutritional practices, including breastfeeding duration, timing of introducing complementary feeding, and protein rich foods[58]. The underlying mechanisms are yet unclear, but there is emerging evidence that it is associated with altered neuro-endocrine programming, and modified by breastfeeding duration and maternal pre-pregnancy overweight[58,59]. Breastfeeding including longer duration of exclusive breastfeeding and longer duration of partial breastfeeding have been associated with a reduced risk of later life obesity and obesity-related complications. Breastfeeding for greater than 40 wk has been associated with lower weight gain by 1 year, and longer duration of breastfeeding with lower odds of developing hypercholesterolemia, hypertension, obesity and type 2 diabetes in later life[60]. Furthermore, mothers who are overweight and obese appear to breastfeed their babies for a shorter duration and introduce complementary foods earlier than mothers of normal weight, which could play a role in their offspring having increased weight and BMI from early childhood[59]. Exclusive breast-feeding for 6 mo or longer, and delaying the introduction of complementary feeding until 5th month of age, are also associated with lower risk of overweight at 5-6 years of age[61]. In addition, social factors including poor nurturing practices and role modeling by parents, early introduction of highly processed high fat, high sugar snacks/meals and exposure to unhealthy food advertising, are early life factors associated with increased offspring obesity.

PREVENTION OF FETAL PROGRAMMING

Primary and secondary prevention of obesity are at the foundation of diabetes prevention programs. While several medical and lifestyle strategies have shown promising effects in slowing progression to and minimizing complications of type 2 diabetes, implementing community measures to prevent obesity/type 2 diabetes are bound to be more cost-effective and beneficial to the community at large, compared to the cost of screening, treating and managing complications of established obesity/type 2 diabetes.

There is now increasing focus on primary prevention of obesity/type 2 diabetes targeting the first 1000-d of life[5]. The first 1000-d of life offers a unique and critical window of opportunity to shape long-term health at the population level, which can have a lasting effect on a country's health and prosperity. Firstly, however, it is prudent to consider the important fundamental question of whether fetal programming of obesity can be minimized by interventions which improve the *in utero* environment of the fetus, in humans. The most promising research findings on preventing adverse fetal programming have come from animal models under experimental conditions. Whether these interventions could be applied in clinical practice, and their effectiveness remain uncertain. However, there are emerging data that improvement in fetal overnutrition and risk of obesity can be achieved *via* maternal interventions. Perhaps the best evidence available to date, is improvement in long-term health outcomes observed in offspring born to severely obese women, after maternal weight loss following bariatric surgery[62]. Studies comparing offspring pairs born to morbidly obese women conceived before and after substantial weight loss following bariatric surgery found that children conceived after surgery had a lower risk of macrosomia (birth weight > 4 kg) at birth, and continued to have better health outcomes in childhood and adolescence including a 50% lower risk of obesity, three-fold lower risk of severe obesity, and better insulin sensitivity and lipid profile, compared to their older siblings[62-64]. These findings confirm that pre-conception weight loss in severely obese mothers can lower fetal overnutrition and reduce the risk of obesity and metabolic complications in the offspring. However, weight reduction by bariatric surgery prior to conception is not an easily available or feasible option for most overweight and obese women of reproductive age, making it necessary to consider alternative interventions which could potentially improve offspring health outcomes.

The World Health Organization, having recognized and acknowledged the potential impact of fetal programming on the obesity epidemic, now advocates a life-course approach for the prevention and control of non-communicable diseases including obesity. This life cycle approach starts with maternal health including preconception, antenatal and postnatal care, and maternal nutrition[65,66]. Potential measures that can be taken at various stages of the life cycle to reduce adverse effects of fetal programming of obesity and type 2 diabetes in future generations are shown in Figure 4.

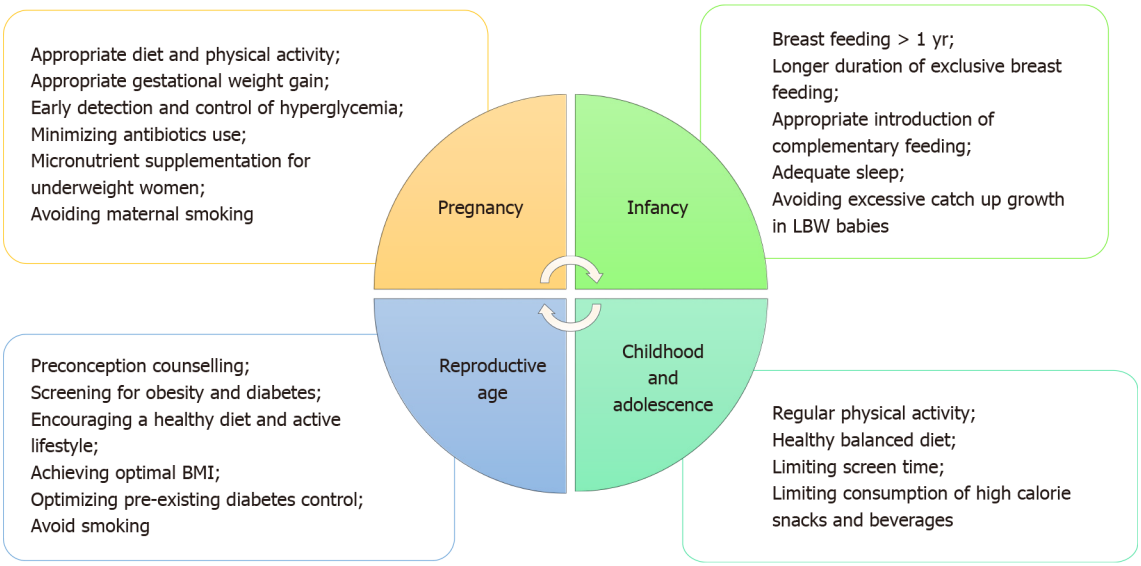
Lifestyle interventions during pregnancy

When considering the impact of intrauterine overnutrition and macrosomia on obesity risk in the next generation, public health measures for healthy maternal weight throughout the reproductive years is justified. Ideally body weight should be optimized to a healthy BMI in all women planning a pregnancy, but this is easier said than done. Pregnancy itself, however, can be an opportune period to commence healthy lifestyle changes, if heath care providers consider it as a "teachable moment" to educate pregnant women on the potential benefits to the baby as well as the mother, and utilize regular and frequent contact with heath care services during this time to provide encouragement and guidance to institute lifestyle interventions[67].

Lifestyle interventions during pregnancy and postpartum appear to reduce gestational weight gain, pregnancy-induced hypertension, need for cesarean section and neonatal respiratory distress syndrome, without any risk of harm to the mother or neonate, across all maternal BMI categories[68]. Thus, a healthy diet and regular exercise for all healthy women during pregnancy and postpartum is a low-cost and feasible intervention which has been advocated as a global health policy[68,69].

Antenatal lifestyle intervention in maternal obesity

Given the rising rates of obesity in women of reproductive age, first in developed, and subsequently in developing countries over the past few decades, there is an urgent need for effective interventions to reduce adverse fetal programming due to maternal obesity[70]. There is expert consensus, that antenatal lifestyle interventions in overweight and obese pregnant women could alter adverse fetal programming and improve offspring health[71,72]. It is postulated that modifying the obesogenic *in utero* environment by lifestyle changes such as increased antenatal physical activity or improved dietary intake during pregnancy could reduce harmful programming effects in the offspring[72]. Antenatal nutritional/lifestyle interventions in overweight/obese pregnant women could potentially be effective by preventing excessive maternal gestational weight gain, and by reducing the risk of developing gestational diabetes, and improving the unhealthy maternal metabolic milieu (insulin resistance, hyperinsulinemia, hyperglycemia, hyperlipidemia, and increased inflammatory markers) which lead to adverse fetal programming[73,74].



DOI: 10.4239/wjd.v13.i7.482

Figure 4 Potential measures that can be taken at various stages of the lifecycle to reduce adverse effects of fetal programming of obesity and type 2 diabetes mellitus in future generations. LBW: Low birth weight; BMI: Body mass index.

Several studies have investigated if lifestyle interventions in overweight and obese mothers during pregnancy can attenuate offspring programming of obesity. In overweight/obese women, multi-component interventions with both a diet and physical activity component have shown some promise, with reduction in gestational weight gain, pregnancy-induced hypertension, macrosomia and neonatal respiratory distress syndrome[68]. Diet was associated with greater reductions in the risk of gestational diabetes mellitus, pregnancy-induced hypertension and preterm birth, compared with any other intervention[68]. However, the effects of these interventions on long-term offspring health are unclear. Studies such as the LIMIT study in Australia reported that providing pregnant women who were overweight or obese with an antenatal dietary and lifestyle intervention improved maternal diet and physical activity during pregnancy, but did not alter 6-mo infant growth and adiposity, or childhood dietary intake, growth and adiposity at 3-5 years of age.

Prenatal exercise has been considered a potential intervention to reduce adverse fetal programming, especially in pregnancies complicated by obesity and/or diabetes[70,75]. Previously, there were concerns regarding the safety of exercise during pregnancy, due to fears regarding teratogenicity from exercise-induced hyperthermia, and fetal hypoxia and intrauterine growth retardation from redistribution of blood flow and nutrients away from the utero-placental circulation during exercise[76]. However, studies on maternal antenatal exercise over the past 20 years have demonstrated that mild-to-moderate intensity antenatal exercise in healthy well-nourished women does not cause observable harm to the fetus[77-80]. There has since been a gradual change of opinion that moderate antenatal exercise is not only safe, but may also be beneficial to offspring health[71,78]. Detailed small scale studies have shown that offspring of physically active lean women who engaged in regular vigorous exercise during pregnancy had lower birthweight and subcutaneous fat at birth, and continued to have lower weight and subcutaneous fat in childhood[81].

However, the effects of antenatal exercise during pregnancy on offspring health appear to vary depending on exercise intensity and frequency, as well as its timing in relation to the period of gestation [82-85]. Commencing exercise in early pregnancy appeared to stimulate feto-placental growth, and increase birth weight, while exercising in the second half of pregnancy appeared to reduce birth weight [83,86]. Furthermore, while it is postulated that regular antenatal exercise during the second half of pregnancy may lead to a reduction in birth weight and adiposity in the offspring, which may be protective against obesity in later life[87], it does not appear to be effective in practice, especially in overweight and obese women[88]. The results of clinical trials targeting antenatal exercise in overweight and obese women have led to varying/inconclusive findings on birthweight and other markers of fetal programming[89,90]. Many trials on supervised antenatal exercise interventions in overweight and obese women have reported a lack of effect on birth weight, or other markers of fetal programming[88, 89,91,92]. One explanation for this could be that obese women, who are generally less physically active, tend to further reduce activity levels during pregnancy[70].

Thus, at present, lifestyle interventions during pregnancy in women with obesity/diabetes have not shown much effect on infant or childhood outcomes. However, many such clinical trials started later in pregnancy, and it is possible that developmental programming occurs much earlier and interventions focusing on healthy lifestyle interventions in pregnant humans are missing the crucial time period for

effectiveness[25].

Interventions for pregnancies complicated by gestational diabetes/pre-existing maternal diabetes

In woman with diabetes in pregnancy, tight glycemic control will help minimize adverse fetal programming of obesity and diabetes in the offspring. For women with both pre-existing and gestational diabetes, offspring outcomes can be optimized by ensuring appropriate gestational weight gain, and optimal glycemic control *via* close monitoring of blood glucose levels, and appropriate medical and nutritional therapy and exercise, throughout the pregnancy[93].

For women with pre-existing type 2 diabetes, insulin has long been considered the gold standard managing diabetes during pregnancy[93]. Careful blood glucose monitoring and titration of insulin doses are important as total daily insulin requirement increases linearly with advancing pregnancy[93]. For women with pre-existing diabetes, it is also important to provide preconception counseling, to achieve optimal pre-conceptional body weight and glycemic control, prior to pregnancy whenever possible. The onus is on health care providers to educate and counsel women with diabetes, particularly on the importance of these aspects not only for their own health status but also to protect their unborn baby from the risks of fetal programming.

For women with gestational diabetes, both insulin and metformin can be used to maintain blood glucose levels if lifestyle interventions are inadequate to achieve adequate glycemic control. Furthermore, for women with a previous history of gestational diabetes, post-partum weight reduction prior to pregnancy could potentially help reduce gestational diabetes mellitus and its associated complications in subsequent pregnancies[94]. The MiG TOFU study, a prospective longitudinal follow-up study in Australia and New Zealand which randomized pregnant women with gestational diabetes mellitus to either metformin or insulin therapy, found that mothers on metformin, had higher glycemia in pregnancy and higher rates of babies with birth weight > 90th percentile, compared to those on insulin therapy, while offspring had similar adiposity at 2 years of age, and similar total and abdominal percentage of body fat and metabolic measures at 7-9 years of age[95].

Interventions for maternal undernutrition

Due to a paucity of evidence from long-term follow-up studies, current recommendations to reduce adverse fetal programming effects of maternal undernutrition, presume that interventions helping to optimize pregnancy outcomes and promote healthy infant growth and development will also help improve the long-term risk of chronic diseases such as central obesity and type 2 diabetes. These recommendations include optimizing maternal nutrition prior to pregnancy, ensuring adequate micronutrient intake in the preconception period and throughout pregnancy before birth, and encouraging breastfeeding and high quality complementary foods to the offspring after birth[96]. Maternal multiple micronutrient supplementation including vitamin and mineral supplementation during the preconception period and early pregnancy have shown some benefit in reducing fetal undernutrition and other adverse fetal programming effects in undernourished mothers[21].

Balanced protein-energy supplementation also appears to be an effective intervention to reduce the prevalence of low birthweight and small-for-gestational-age births, especially in undernourished women[97]. Thus, ensuring appropriate and adequate intake of micronutrients, essential fats and protein supplementation in mothers with undernutrition during pregnancy, could improve the nutritional condition of the mother, and confer a protective benefit to the offspring by reducing fetal growth restriction and low birth weight in developing countries with high rates of maternal undernutrition[96].

Interventions in offspring in infancy and childhood

Intervention strategies to reduce adverse effects of fetal programming of later life obesity may be more effective if they target multiple modifiable factors, focusing on the first 1000-d of life.

Breastfeeding appears to protect against obesity in childhood, and could be a modifying factor to mitigate the adverse effects of fetal programming *in utero*. Promoting longer duration of full breastfeeding and partial breastfeeding, and delaying the introduction of complementary feeding could protect the offspring from obesity. Exclusive breastfeeding for 6 mo or longer, and delaying the introduction of complementary feeding until 5th month of age, has been associated with a lower risk of overweight at 5-6 years of age[61]. The protective effects of breastfeeding on the offspring of diabetic mothers in very early life appears somewhat conflicting, with one study suggesting a potential negative long-term influence on the risk of becoming overweight in offspring exposed to breast milk from mothers with diabetes (type 1 or gestational diabetes) during the first week of life[60]. However, overall, the benefits of breastfeeding appear to be beneficial, and protect infants from the adverse effects of fetal programming of obesity and type 2 diabetes. Women who were overweight or obese before pregnancy, appear to breastfeed their offspring for a shorter time and introduce complementary feeding earlier than normal weight women, which could contribute towards their children being heavier and having a higher BMI by end of infancy[59]. Thus, it is especially important to take measures to encourage and support longer duration of breastfeeding in women who are overweight or obese.

Further protective measures that could be helpful in optimizing long-term health during infancy include ensuring adequate sleep and minimizing antibiotic use. Early antibiotic use before 2 years of age has been associated with disruption of the gut microbiota, and a higher risk of childhood overweight and obesity[98]. Recent evidence on the associations with gut microbiota and infant weight gain or child weight status, suggest that dietary manipulation with human milk and pre/probiotic formulations holds promise for preventing obesity[99].

Furthermore, as short sleep duration increased the risk of childhood obesity, public health efforts that encourage children to have sufficient sleep time are also important in combating obesity[100]. Project Viva prospectively studied the cumulative number of modifiable early-life risk factors associated with programming of obesity/type 2 diabetes in mother-offspring pairs including: maternal smoking and consumption of high sugar-sweetened beverages during pregnancy, excessive gestational weight gain; breastfeeding for less than 1 year; complementary food introduction before 4 mo; and infant sleep duration less than 12 h daily. When reassessed in early adolescence, they found that offspring with 5-6 risk factors had a 2.5 higher rate of obesity and metabolic syndrome, compared to those with 0-1 risk factors[101]. Thus, it appears that promoting exclusive breastfeeding for at least the first 4 mo of life, and continuation of breastfeeding beyond the first year of life, as well as ensuring adequate sleep for infants, could potentially reduce the risk of further life obesity in infants who have already been exposed to risk factors for adverse fetal programming *in utero*.

Other potential strategies to reduce the adverse impact of fetal programming include identifying and targeting young children at higher risk of fetal programming of obesity/diabetes such as offspring of mothers with obesity/diabetes/undernutrition during pregnancy, especially those being reared in highly urbanized obesogenic environments, for healthy lifestyle interventions during early childhood to encourage them towards a healthier lifestyle, and prevent adverse metabolic health outcomes in later life[46]. Pairing breastfeeding with healthy weaning foods is likely to promote healthy weight trajectories.

A recent review reported that several multicomponent trials promoting breastfeeding, responsive feeding, and a healthy diet (increased fruit and vegetables, and limiting sugar sweetened beverages and unhealthy snacks) through home visits or education at baby health clinics over 1-2 years duration, showed relative reductions in BMI in offspring at the end of the intervention, although early benefits were not maintained in the two trials reporting follow-up 1 year to 3 years later[102]. Thus, there is some evidence that nutrition or feeding interventions in the first two years of life can have a positive impact on a child's BMI, but maintaining this benefit may require continued intervention and sustainable environmental change[102].

Observational studies suggest that rapid weight gain in infancy also increases the long-term risk of obesity and type 2 diabetes in infants from both low-and high-income countries, among infants born preterm or at term, with normal or low birth weight for gestation[103]. Furthermore, it has been hypothesized that the increased risk of adverse long-term outcomes including central obesity and type 2 diabetes in low birth weight infants may be driven by accelerated postnatal catch-up growth. While some studies on health outcomes in babies with low birth weight have reported that increased catch up growth was associated with higher BMI or higher serum cholesterol levels in early adolescence, the quality and quantity of the evidence is limited[102]. Thus, it is prudent to recommend "striking a healthy balance", especially for low and middle income countries, until more information on underlying mechanisms and suitable interventions on minimizing adverse effects of catch up growth in low birth weight infants become available[104].

Beyond infancy, promising interventional approaches for pre-school age children include age appropriate health and nutrition education for preschoolers, combined with teaching parents behavioral change strategies and increasing parenting skills[105]. For school children, school-based interventions have been shown to be effective in reducing excessive weight gain in children[106]. Programs involving both school and family and lasting ≤ 1 year were the most efficacious for primary school children aged between 6 and 12 years; while family-based interventions have been effective in children < 6 years old [107].

Preconception care

Healthy lifestyle behaviors during the preconception period are important to optimize maternal and child outcomes. Community nurses and midwifery professions who are active across both preconception and pregnancy could play an important role in such interventions[108]. Many women of reproductive age do not appear to have optimal preconception lifestyle behaviors, and a recent systematic review identified the absence of knowledge on healthy behaviors as the most common barrier[109]. The need for further studies on how to best improve preconception women's capability, opportunity, and motivation to modify their lifestyle behaviors has been emphasized[109]. At present, there is a lack of international consensus guidelines on weight management preconception, and its impact on fertility, pregnancy and subsequent maternal and infant outcomes.

The reversibility of obesity-induced parental programming has only recently received attention. These programmed changes to offspring health may be partially restored *via* diet/exercise interventions in obese fathers, prior to conception, *via* improvement in sperm DNA integrity. Promising results in animal models utilizing diet and exercise interventions have shown improvements in sperm function

and molecular composition, resulting in restorations of both embryo and fetal health and subsequent male offspring fertility[57]. However, it is noteworthy, that most data surrounding paternal obesity and offspring phenotypes have come from rodent models, and implications for clinical practice warrants further research[57].

CHALLENGES AND THE WAY FORWARD

Maintaining a healthy maternal BMI and lifestyle from preconception and throughout pregnancy will help minimize the risk of future obesity in the offspring. A balanced diet with low glycemic load, and light-to-moderate intensity physical activity for 30-60 min daily, at least for 3-5 d per week is recommended[68]. Maternal pre-pregnancy and early pregnancy metabolic conditions often programs early placental function and gene expression in the first trimester of pregnancy, prior to when most intervention trials are initiated[110]. Interventions commenced during pregnancy have met with limited success in preventing adverse fetal programming effects. This could be because most interventions were instituted after the first trimester, where it may have been too late to have a positive impact on fetus programming.

Given the widespread and long-lasting impact of adverse fetal programming, a population-based life-course approach is warranted, until more focused and specific ways to prevent adverse fetal programming are discovered. As the evidence on the peri-conceptional environment on offspring long-term health is compelling, updated guidelines and guidance for parental preparation for pregnancy, prior to conception to protect the health of offspring is required[26,110]. This should be followed by proper guidance for parents regarding appropriate nutrition, physical activity, and screen time in early childhood. Furthermore, school based interventions with family involvement could be effective in improving dietary habits and lifestyle in primary school children[111]. Whole community interventions addressing both policy and behavior change are needed[112]. Wider dissemination of health messages advocating healthy lifestyle as a means of providing a better chance of a healthier life for future generations is recommended[112].

CONCLUSION

Fetal programming is an important contributor to the global obesity epidemic. Risk factors for adverse fetal programming include maternal obesity, diabetes, undernutrition, smoking, stress, operative delivery and use of antibiotics in pregnancy, as well as paternal factors including over/undernutrition. These factors lead to fetal programming *via* multiple complex pathways including alterations in organ formation and homeostatic pathways, epigenetic changes, and changes in gut microbiota. Specific mechanistic pathways are still being unraveled. Using this knowledge to find effective and feasible methods of preventing adverse fetal programming is an issue of global importance. The current state of knowledge dictates that future research should be directed towards earlier interventions starting in the pre-conceptional period. Until such time, a multi-pronged life-course approach, focusing on maternal health, antenatal and postnatal care, as well as healthy lifestyle interventions for preschoolers, school children, and young adults of reproductive age is advocated.

ACKNOWLEDGEMENTS

We wish to acknowledge Dr. Jayakody C and Dr. Hewakuruppu S for their assistance with development of the figures for this manuscript. We are grateful to Dr. Perera BJC, Joint Editor of The Sri Lanka Journal of Child Health, for editing the final manuscript.

FOOTNOTES

Author contributions: Seneviratne SN and Rajindrajith S conceptualized and designed this manuscript; Seneviratne SN reviewed the literature and wrote the manuscript; both authors read and approved the final manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Gao CC
L-Editor: Webster JR
P-Editor: Gao CC

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 498-520

DOI: 10.4239/wjd.v13.i7.498

ISSN 1948-9358 (online)

REVIEW

Biochemical composition of the glomerular extracellular matrix in patients with diabetic kidney disease

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Specialty type: Medicine, general and internal

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bayoumi RAL, United Arab Emirates; Javor E, Croatia

Received: February 1, 2022
Peer-review started: February 1, 2022
First decision: April 18, 2022
Revised: April 19, 2022
Accepted: June 24, 2022
Article in press: June 24, 2022
Published online: July 15, 2022

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Abstract

In the glomeruli, mesangial cells produce mesangial matrix while podocytes wrap glomerular capillaries with cellular extensions named foot processes and tether the glomerular basement membrane (GBM). The turnover of the mature GBM and the ability of adult podocytes to repair injured GBM are unclear. The actin cytoskeleton is a major cytoplasmic component of podocyte foot processes and links the cell to the GBM. Predominant components of the normal glomerular extracellular matrix (ECM) include glycosaminoglycans, proteoglycans, laminins, fibronectin-1, and several types of collagen. In patients with diabetes, multiorgan composition of extracellular tissues is anomalous, including the kidney, so that the constitution and arrangement of glomerular ECM is profoundly altered. In patients with diabetic kidney disease (DKD), the global quantity of glomerular ECM is increased. The level of sulfated proteoglycans is reduced while hyaluronic acid is augmented, compared to control subjects. The concentration of mesangial fibronectin-1 varies depending on the stage of DKD. Mesangial type III collagen is abundant in patients with DKD, unlike normal kidneys. The amount of type V and type VI collagens is higher in DKD and increases with the progression of the disease. The GBM contains lower amount of type IV collagen in DKD compared to normal tissue. Further, genetic variants in the α3 chain of type IV collagen may modulate susceptibility to DKD and end-stage kidney disease. Human cellular models of glomerular cells, analyses of human glomerular proteome, and improved microscopy procedures have been developed to investigate the molecular composition and organization of the human glomerular ECM.

Key Words: Diabetes; Kidney disease; Glycosaminoglycans; Factor H; Sialic acid; Laminin; Collagen; Fibronectin-1; Extracellular matrix

Core Tip: Diabetic kidney disease is associated with profound disturbance in glomerular extracellular matrix (ECM). Understanding the mechanisms that regulate glomerular ECM synthesis and repair may contribute to design therapeutic strategies that improve clinical outcomes. The cytoskeleton inside the foot processes of podocytes is connected to the glomerular basement membrane (GBM) *via* associated proteins. There is a reciprocal interaction between the cellular cytoskeleton and the extracellular tissue that contribute to regulate ECM composition. Loss of anchor points in the GBM may lead to podocyte detachment. Likewise, alterations in the podocyte cytoskeleton may unfasten the cell and impair the filtration barrier.

Citation: Adeva-Andany MM, Carneiro-Freire N. Biochemical composition of the glomerular extracellular matrix in patients with diabetic kidney disease. *World J Diabetes* 2022; 13(7): 498-520
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/498.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.498

INTRODUCTION

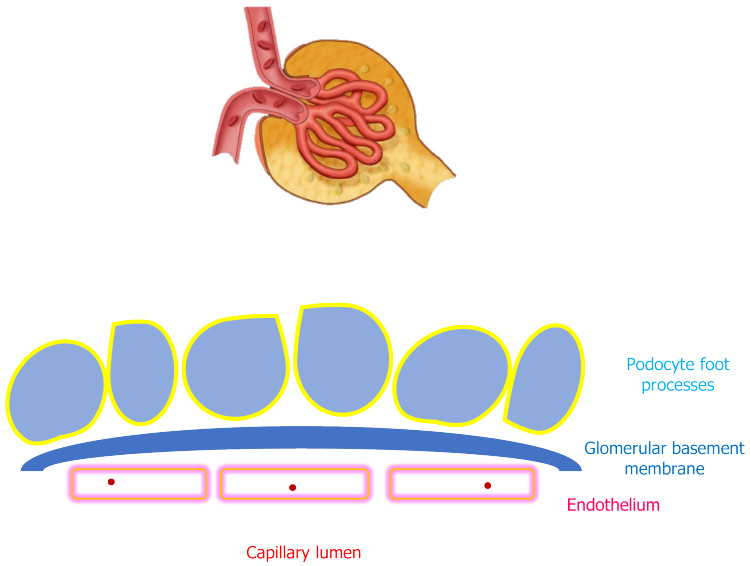
In human glomeruli, mesangial cells (which are thought to be akin to vascular smooth muscle cells) produce mesangial matrix while podocytes wrap glomerular capillaries with cellular extensions named foot processes and tether underneath glomerular basement membrane (GBM) (Figure 1). The turnover of the GBM present at birth and the ability of adult podocytes to restore damaged GBM are unclear. These cells have limited proliferation capacity, but they can undergo hypertrophy to compensate for the detachment and loss of contiguous podocytes, thus avoiding uncovered GBM areas to preserve the filtration barrier. Podocyte detachment may be caused by an altered composition of the GBM with deficiency of anchor points or by anomalies in the connection apparatus that links the foot processes to the GBM. The actin cytoskeleton is a major cytoplasmic component of the podocyte foot processes and connects the cell to the GBM. Actin-associated proteins such as α-actinin-4 and inverted formin-2 attach the actin cytoskeleton to plasma membrane components (such as integrins, syndecans and dystroglycans), which in turn bind to their ligands in the GBM, including laminin and fibronectin-1[1-4]. The integrity of the GBM is crucial to maintain the filtration barrier, as highlighted by the clinical consequences of disorders that alter GBM components, such as laminin or collagen. Diabetes and other conditions associated with insulin resistance (such as Alström syndrome) are associated with a systemic and pronounced alteration in the composition of extracellular matrix (ECM), including the kidney and the blood vessels, that leads to multi-organ interstitial fibrosis[5]. Pathogenic mechanisms underlying this disturbance are unclear. Understanding the pathways of ECM assembly and remodeling and the cell-ECM interactions is crucial for designing therapeutic strategies and tissue engineering. A growing number of procedures have been improved and developed to investigate the biochemical composition and architecture of the ECM and its mutual interaction with the contiguous cells. Among them are biochemical assays to identify and quantify ECM components, genetic methods to investigate gene expression, imaging procedures, human cell cultures, and *in vitro* pharmacological evaluations to assess metabolic pathways.

Nuclear magnetic resonance spectroscopy and soft-ionization mass spectrometry represent complimentary techniques for ECM research. Mass spectrometry techniques (such as matrix-assisted laser desorption and ionization) are useful for compositional analysis whereas nuclear magnetic resonance spectroscopy evaluates the molecular architecture of the ECM and its dynamics[6]. Raman spectroscopy is a label-free vibrational technique that contributes to characterize the molecular ECM structure and composition[7,8].

Histological methods for ECM analysis with conventional microscopy include immunohistochemistry and zymography. The former can be utilized to determine the localization of various ECM proteins while the latter may be used to evaluate proteinase activity in the ECM. In addition, imaging methods have been designed to characterize the human ECM and the adjacent cells at the molecular and cellular level. Scanning electron microscopy and multi-harmonic generation microscopy can be used to visualize ECM components and assess their structural properties[9]. Multiphoton imaging has been described to analyze the human structural organization of elastin and collagen during mechanical loading[10].

The construction of flat and tubular collagen gel-based scaffolds cellularized with vascular smooth muscle cells have enriched vascular tissue engineering[11]. Microgel assembly, a macroscopic aggregate formed by assembly of microgels, can be applied to tissue engineering and cell cultures[12].

A variety of genetic techniques are useful on ECM research. MicroRNAs are noncoding RNAs that regulate gene expression and participate in ECM pathophysiology. Microarrays can be used to determine microRNA profiles[13]. Weighted gene co-expression network analysis enables the identification of clusters of related genes that can be associated with specific clinical phenotypes. This technique has been used to assess differentially expressed ECM genes in patients with diabetic kidney



DOI: 10.4239/wjd.v13.i7.498

Figure 1 Glomerular capillaries and glomerular basement membrane.

disease (DKD) and other glomerular diseases[14,15]. The Matrisome Project has been developed to characterize genes encoding structural and associated ECM proteins (http://matrisomeproject.mit.edu).

Human cell culture technology and pharmacological investigations on cultured cells are instrumental tools on ECM research. Human pluripotent stem cells in culture have been used to generate models of various tissues. The presence of ECM in the cultures provides cues to the cells that modify their behavior and improves similarity to the native human tissue, including the kidney[16]. Stem cells reside in "niches" within the ECM and the composition of the ECM contributes to define the degree of quiescence and turnover of these cells[17]. Manufacture of a suitable ECM is crucial to alter growth, differentiation, and proliferation of stem cells and use them for tissue engineering[18]. Three-dimensional decellularized ECM derived from mesenchymal stem cell cultures has been attained by application of macromolecular crowding[19]. Three-dimensional tissue constructs that recapitulate human fibrous connective tissue have been achieved by using cultures of primary human fibroblasts, enabling the quantification of cell-derived changes in ECM synthesis in response to several stimuli, such as nutrient composition or pharmacological compounds[20]. Treatment of human bone marrow-derived mesenchymal cells with high molecular weight hyaluronic acid increases fibronectin production and ECM deposition, suggesting that hyaluronic acid-based biomaterials may be useful to promote ECM formation[21]. A pulsatile perfusion culture of progenitor cells has been developed as an *in vitro* system to construct vascular tissue[22]. Cell-matrix interactions may be investigated by micro-electro-mechanical systems and Organ-on-a-Chip technology[23].

In the kidney, human cellular models of glomerular epithelial cells have been developed that can be used to evaluate podocyte pathophysiology and investigate therapeutic strategies[24]. Investigations of the glomerular proteome have provided information on the proteins expressed in the glomerular ECM of adult normal human kidney. A database has been created that may be used for clinical research on the pathophysiology of kidney diseases[25-27]. Proteomic analysis of human glomerular ECM may be conducted from sections retrieved by kidney biopsy samples[28]. The sub-diffraction resolution stochastic optical reconstruction microscopy (STORM) facilitates the investigation of the molecular organization within the human GBM[29].

NORMAL COMPOSITION OF THE HUMAN GBM AND MESANGIAL MATRIX

The analysis of the specific composition of the GBM and mesangial matrix is hindered by the technical obstacle of adequately separate these two compartments of glomerular ECM, as procedures that isolate glomerular ECM achieve samples that contain both GBM and mesangial matrix. However, immunohistochemical analyses contribute to determine the differential constitution of the two structures (Table 1)[27,30-32]. Major components of the normal glomerular ECM are laminin and collagen. In addition, glycosaminoglycans, proteoglycans, sialic acid, and fibronectin-1 are important constituents of the kidney ECM in humans.

Table 1 Major components of the glomerular basement membrane and the mesangial matrix in normal human glomeruli

	Glomerular basement membrane	Mesangial matrix
Heparan sulfate proteoglycan	Abundant	Abundant
Laminin	Major component	Minor component
Fibronectin	Minor component	Major component
Type I collagen	Absent in most studies	Absent in most studies
Type III collagen	Absent in most studies	Absent in most studies
Type IV collagen	Major component	Present (inconsistent amounts)
Type V collagen	Present	Present
Type VI collagen	Present	Present
Type XVII collagen	Present	Unknown
Type XVIII collagen	Present	Present
Tubulointerstitial nephritis antigen-like-1	Low abundance	High abundance
Nidogen / Entactin	Present	Low abundance
Fibulin-1	Present	Present
Fibrillin-1	Present	Present
Nephronectin	Present	Present
Vitronectin	Absent	Present
Microfibril-associated proteins	Absent	Present

Glycosaminoglycans, proteoglycans, and sialic acid in the normal glomerulus

Proteoglycans consist of a core protein attached to one or more glycosaminoglycan chains, which are formed by linear polysaccharides. Sulfate groups are usually bound to the unbranched polysaccharide chains, creating a high negative charge[33,34].

In kidney specimens from healthy humans, heparan sulfate is the predominant glycosaminoglycan present in the glomerular ECM, followed by hyaluronate, dermatan sulfate, and chondroitin sulfate isomers 4-sulfate and 6-sulfate[35-37].

Immunohistochemical studies show that both GBM and mesangial matrix contain heparan sulfate proteoglycans[33,38-40]. Among them, mass spectrometry-based analyses of normal human kidney samples reveal that agrin and perlecan are present in the glomerular proteome[27]. Agrin is a major heparan sulfate proteoglycan present in human GBM. Immunoelectron microscopy shows a linear distribution of agrin throughout the width of the normal GBM. In addition to the GBM, agrin mRNA and protein are detected in normal lungs[34,41]. Localization of agrin to the human GBM has been confirmed by STORM. Using this procedure, agrin is predominantly detected at the epithelial surface compared to the endothelial aspect of the GBM[29]. The precise function of agrin in the human kidney has not been defined, but it may contribute to the adhesion of the GBM to the podocyte by tethering laminins to cell surface receptors such as integrins or α-dystroglycan[34,41]. In normal human kidney specimens, perlecan stained the GBM only slightly, in contrast to the strong staining of the mesangium, the Bowman's capsule, and the tubular basement membrane. The function of this heparan sulfate proteoglycan in the normal kidney remains to be clarified[34,42,43]. Unlike agrin, immunoelectron microscopy shows that perlecan is distributed only on the endothelial side of the GBM[34].

Sialic acid (neuraminic acid) is a nine-carbon carbohydrate that may exist as several derivatives. In humans, the most common sialic acid byproduct is the acetylated compound N-acetyl-neuraminic acid. Sialic acid typically occupies the terminal domain of oligosaccharide chains of some glycolipids and glycoproteins and usually protrudes from the cell surface. Sialidases (neuraminidases) are enzymes that remove sialic acid residues from glycosaminoglycans attached to proteins or lipids on the cell surface (desialylation). Sialyltransferases catalyze the addition of sialic acid residues to glycosaminoglycans (sialylation). Sialylated conjugates are identified by specific binding to lectins or by cationic dyes such as alcian blue[44,45]. In normal human kidney specimens, sialic acid stains strongly the podocytes, unlike glomerular capillaries and Bowman's capsule[44].

Both sulfated glycosaminoglycans and sialic acid are polyanions that have an essential role in the identification of "self" structures to avoid complement activation and subsequent complement-mediated injury in host tissues[46-49]. Sulfation can occur at various positions within the glycosaminoglycan structure creating the potential for high molecular variability. The unique position of sulfate groups in the glycosaminoglycan molecule is named sulfation code and defines functional character-

istics of the sulfated glycosaminoglycan, such as its interaction with proteins. Hyaluronic acid is a glycosaminoglycan that lacks sulfate groups and is not attached to a protein core to form proteoglycans [50]. Factor H is a glycoprotein that inhibits the alternative pathway of complement in "self" structures (as opposed to foreign elements such as pathogens), by recognizing sialic acid or sulfated glycosaminoglycans present on "self" biological surfaces. The interaction between factor H and sulfated glycosaminoglycans is highly specific and depends upon the sulfation code. Little or no binding occurs with hyaluronic acid. The binding of factor H to sialic acid or sulfated glycosaminoglycans on biological surfaces protects the host from autolytic complement attack (Figure 2). Deficit of binding sites for factor H due to loss of sulfated glycosaminoglycans or sialic acid (or alteration of the sulfation code or the sialylation pattern) impairs factor H binding to "self" structures and may result in complement-mediated damage due to unrestrained activation of the alternative pathway of complement[46,48,49,51-54].

Laminin in the normal glomerulus

Protein quantification of the glomerular ECM proteome by mass spectrometry reveals that laminin isoforms and type IV collagen are the most abundant proteins in the glomerular ECM[27]. Laminins are heterotrimeric proteins composed of α, β, and γ glycoprotein chains. Different α, β, and γ chains create diverse isoforms of laminin heterotrimers, such as laminin α5/β2/γ1 (laminin 521). Laminin heterotrimers polymerize in the extracellular space to form a network. Laminin polymerization is required for initiation of basement membrane formation. The actin cytoskeleton plays an important role in extracellular laminin polymerization. *In vitro* studies using myotubes reveal that the organization of extracellular laminin into networks is abnormal when the actin cytoskeleton is disrupted with cytochalasin (an agent that prevents actin polymerization) compared to control myotubes free of this compound. Cytochalasin-treated myotubes show no arrangement of surface laminin into complex networks, unlike control myotubes that show normal laminin array. However, no detrimental effect on laminin network formation was observed with wortmannin, an inhibitor of phosphatidylinositol 3-kinase[55].

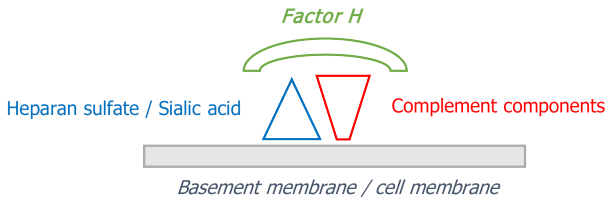
Laminin isoforms has been identified in the mesangial matrix of normal human kidneys, although this protein is predominantly detected in the GBM. Immunohistochemical studies show a continuous staining for laminin in the normal GBM. Immunogold electron microscopy reveals that laminin is distributed throughout the entire thickness of the GBM[37-40,56]. The organization of laminin 521 in normal human GBM has been investigated with STORM. Laminin 521 (and agrin) have their N-terminal domains facing the interior of the GBM while their C-terminal domains are oriented towards the surface of endothelial cells and podocytes[29].

The important functional role of laminin in the glomeruli is underlined by the clinical consequences of genetic mutations that alter the protein. Mutations in the gene that codes the β2 chain of laminin cause an autosomal recessive clinical spectrum of disorders that ranges from isolated congenital nephrotic syndrome (type 5) to Pierson syndrome, which consists of a combination of ocular abnormalities, neurological manifestations due to defects of the neuromuscular junction, and congenital nephrotic syndrome with diffuse mesangial sclerosis progressing rapidly to end-stage kidney disease (ESKD)[57,58].

Fibronectin-1 in the normal glomerulus

Fibronectin-1 is a dimeric glycoprotein circulating in normal plasma and present in the healthy human kidney. Immunohistochemical studies show that fibronectin-1 is mainly present in the mesangium and to a far less degree in the GBM. Staining for fibronectin-1 also occurs in the Bowman's capsule and the peritubular interstitium[27,37,39,40,59,60]. The function of fibronectin-1 in the kidney is largely unknown. *In vitro* studies using cultured fibronectin-null cell lines find that fibronectin-1 polymerization in the ECM is involved in the deposition of other ECM components, such fibulin, type III collagen, and type I collagen[61]. Fibronectin-1 possesses several domains that may function as binding sites for other molecules, including collagen and cell surface proteins such as integrins. Fibronectin-1 may connect to plasma membrane proteins which in turn are linked to the intracellular actin cytoskeleton. There is a reciprocal relationship between fibronectin-1 and the actin cytoskeleton. Agents that disrupt actin polymerization block the extracellular organization of fibronectin-1 into a network. In turn, inhibition of fibronectin-1 polymerization in the ECM induces changes in the actin cytoskeleton [62]. *In vitro* studies using cultured human podocytes show that fibronectin-1 is essential for the attachment of podocytes to the GBM during mechanical stress. Mechanical stretch induces a marked upregulation of fibronectin-1 in normal podocytes. Accordingly, in podocyte cell lines lacking fibronectin-1, a loss of podocytes greater than 80% is observed following mechanical stress[4].

An abnormal glomerular accumulation of fibronectin-1 may occur in acquired disorders, such as DKD and other diseases that feature mesangial expansion, such as lupus nephritis, IgA nephropathy, and membranoproliferative glomerulonephritis[62-65]. In addition, glomerulopathy with fibronectin-1 deposits (fibronectin nephropathy) is an autosomal dominant disease characterized by deposits of fibronectin-1 in the mesangial matrix and subendothelial space. Mutations in the *FN1* locus (that encodes fibronectin-1) at 2q32 have been identified as the genetic cause of the disease[63]. Clinical manifestations include proteinuria, hematuria, hypertension, and kidney failure that may progress to



DOI: 10.4239/wjd.v13.i7.498

Figure 2 Binding of complement factor H to components of the alternative pathway of complement (C3b) and heparan sulfate/sialic acid on "self" structures (cell membranes or basement membranes).

ESKD. Asymptomatic patients harboring *FN1* mutations have been documented. Light microscopy reveals enlarged glomeruli with deposits of eosinophilic material in the mesangium and subendothelial space that shows reactivity with periodic acid Schiff (PAS) and trichrome stains while methenamine silver and Congo red stain negative. No immunoglobulin or complement factors are detectable by immunofluorescence studies. Electron microscopy reveals a normal GBM and large electron-dense deposits in the mesangium extending to the subendothelial space. Diagnosis can be established by specific immunohistochemical analysis, as the glomerular deposits stain intensely with anti-fibronectin-1 antibodies (Table 2)[62-65].

Collagen in the normal glomerulus

Initial studies suggested the presence of collagen in normal human glomerular ECM by the high amount of glycine, hydroxyproline, and hydroxylysine in glomerular extracts[30,66-68]. Relative protein quantification confirms an abundant amount of type I, type IV, and type VI collagen in human glomerular ECM. As mentioned, type IV collagen and laminin are the most abundant proteins in the normal glomerular ECM[27].

Type I collagen in the normal glomerulus: Some studies fail to find type I collagen in normal human glomerular ECM, either the GBM or the mesangium[39,40,69]. However, mass spectrometry performed in adult kidney samples identifies abundant type I collagen in the glomerular ECM, although its localization to a specific glomerular ECM sector (GBM, mesangial matrix, or other) is undefined[27].

Type III collagen in the normal glomerulus: Type III collagen mRNA or protein have not been detected in healthy human glomeruli. Neither the mesangial matrix nor the GBM normally possess type III collagen[33,38-40,69-71]. However, type III collagen has been observed in sclerotic glomeruli, suggesting that production of this collagen type is linked to the progression of glomerular sclerosis[69]. In addition, glomerular type III collagen has been demonstrated in human kidney diseases, such as DKD, LIM homeodomain transcription factor-1β (LMX1β)-associated nephropathy (LAN) and type III collagen glomerulopathy.

Heterozygous loss of function mutations in the *LMXIβ* gene (located on chromosome 9q34) cause LAN. Patients with LAN may present with isolated nephropathy or may exhibit additional extrarenal clinical manifestations composing the nail-patella syndrome[72,73]. The LMXIβ protein is a transcription factor that possesses two LIM domains (cysteine rich sequences that usually mediate protein-protein interactions) and a homeodomain that regulates target gene transcription. The precise role of the LIM-homeodomain protein LMXIβ in humans remains unknown[74,75]. Nail-patella syndrome or onycho-osteodysplasia is characterized by the association of nail hypoplasia or dysplasia, bone abnormalities that affect the knees, elbows, and pelvis, glaucoma, sensorineural hearing impairment, and nephropathy. Renal manifestations include hematuria, proteinuria, and kidney failure that may evolve to ESKD. *LMX1β* mutations may also cause isolated autosomal dominant kidney involvement with no extrarenal manifestations[72,73,75-78]. LAN is characterized by deposition of type III collagen within the GBM on electron microscopy examination. Fibrillar type III collagen bundles may be seen occasionally in the mesangial matrix as well. The GBM may demonstrate focal irregular thickening, thinning, splitting, or wrinkling and may contain patchy electron-lucent ("moth-eaten") areas. Hyperplasia and effacement of podocyte foot processes is usually observed. The basement membrane of kidney tubules appears markedly thickened and demonstrates type III collagen deposition[78,79]. Light microscopy examination may reveal focal segmental glomerulosclerosis (FSGS) or unremarkable findings, such as mild interstitial fibrosis or mesangial proliferation. Immunofluorescence microscopy yields negative or non-specific findings, such as slight granular deposits of C3 in the mesangium. Specific immunohistochemical analyses show that the fibrillar material present within the GBM (and occasionally the mesangial matrix) is type III collagen[78-82]. The histological phenotype of LAN is expanding, as heterozygous mutations in the *LMXIβ* gene have been reported in patients with autosomal dominant FSGS without ultrastructural abnormalities of the GBM and in families with FSGS and myelin figures and zebra bodies (electron-dense multilamellar inclusions) in podocytes, mesangial

Table 2 Staining characteristics of the mesangial deposits in diabetic kidney diseases, fibronectin-1 nephropathy, and type III collagen glomerulopathy

	Periodic acid Schiff	Methenamine silver	Congo red	Specific analysis
Diabetic kidney disease	Positive	Positive	Negative	Unknown material
Fibronectin-1 nephropathy	Positive	Negative	Negative	Fibronectin-1
Type III collagen nephropathy	Negative	Negative	Negative	Type III collagen

cells, and tubular epithelium. Patients affected with LAN and myelin figures and zebra bodies are free of Fabry's disease, which is the typical cause of these inclusions. Therefore, *LMX1β* pathogenic variants should be ruled out as a potential cause of autosomal dominant kidney disease, sporadic and hereditary forms of FSGS, and steroid-resistant nephrotic syndrome, regardless of extrarenal manifestations. In addition, the presence of myelin figures or zebra bodies may hint toward LAN diagnosis in patients free of lysosomal storage disorders or drug-induced phospholipidosis, although the mechanism underlying the appearance of these structures in LAN is unclear[80,82,83].

Type III collagen glomerulopathy (collagenofibrotic glomerulopathy) is characterized by deposition of type III collagen fibrils within the mesangial matrix and along the subendothelial aspect of a normal GBM. The cause of the excessive production and deposition of type III collagen in the glomeruli is unknown. The diagnosis of the disease is confirmed by electron microscopy and specific immunohistochemistry demonstrating the presence of mesangial type III collagen. Light microscopy reveals diffuse mesangial expansion that cause glomerular enlargement. In the advanced stage, the expanded mesangium shows a lobular appearance reminiscent of Kimmelstiel-Wilson nodules of DKD. In addition, the subendothelial accumulation of this material causes double contour or "reduplication" of the GBM. Unlike DKD, the amorphous material present in the mesangium stains negative with PAS. Masson's trichrome stain identifies the blue-colored collagen within the mesangium. Immunofluorescence microscopy studies are negative for immunoglobulins and complement components. Immunohistochemistry reveals strong staining for antibodies to type III collagen in the widened mesangial and subendothelial areas. Electron microscopy reveals a normal GBM and confirms the accumulation of electron-dense fibrillar material consistent with dense collagen bundles in mesangial and subendothelial zones. The fibrils exhibit a transverse band structure with distinctive periodicity suggesting type III collagen fibers. The mesangium and subendothelium acquire a mottled appearance due to the presence of collagen fibrils[84-86]. Accumulation of mesangial type III collagen has been reported in one patient with inherited factor H deficiency[87].

Type IV collagen in the normal glomerulus: Type IV collagen is an abundant protein of the glomerular ECM and may be observed in the GBM and the mesangium. In the normal GBM, a distinct continuous staining for type IV collagen indicates that this collagen type is a predominant component[27,31,37,39,71,88].

The molecule of type IV collagen consists of three α chains. Six genes (*COL4A1-6*) encode six different α chains that create several isoforms of type IV collagen. The α3, α4, and α5 chains of type IV collagen contain more cysteine than the chains α1 and α2. Therefore, α1α1α2 (α112) trimers possess fewer disulfide bonds than α3α4α5 (α345) heterotrimers. The relative protein abundance of the α1 and α2 chains in normal adult glomerular ECM has been reported higher compared to the richness of the α3, α4, and α5 chains[27].

Ultrastructural examination with immunogold technique reveals that type IV collagen is concentrated in the endothelial zone and decreases towards the epithelial third of the GBM in normal human glomeruli. In addition, the α1 chain is distributed primarily along the endothelial side of GBM whereas the α3 and α4 chains are seen throughout the thickness of the GBM[56,89]. Kidney assessment with STORM reveals that type IV collagen α345 trimers are localized at the center of the GBM while type IV collagen α112 trimers are located to the endothelial side. Co-labeling for both trimers of type IV collagen (α345 and α112) suggest the α112 network occupies the space between the central α345 Layer and the endothelial surface of the GBM[29].

In addition to the GBM, type IV collagen is detectable in the mesangial matrix of normal human glomeruli[38-40,43,71]. Investigations using quantitative immunogold electron microscopy show that mesangial type IV collagen labeling appears uniform throughout the mesangial matrix and extends to the subendothelial side of the GBM[56,89]. Electron microscopy examination with immunogold technique shows that the α1 chain of type IV collagen is distributed primarily along the mesangial matrix and the endothelial side of GBM whereas the α3 chain of type IV collagen is not detected in normal human mesangial matrix[32].

The relevance of type IV collagen to kidney structure and function is highlighted by the clinical consequences of mutations in genes that code the α chains of this collagen type. Mutations in the *COL4A3-5* genes cause type IV collagen-related kidney disease (Table 3). The *COL4A5* gene encodes the α5 chain and maps to the X chromosome. Mutations in this gene account for X-linked Alport syndrome.

Table 3 Type IV collagen-related kidney disease

	Gene/location	Protein	Mutation	Risk of progression to end-stage kidney disease
X-linked Alport syndrome	*COL4A5*/X chromosome	α5 chain of type IV collagen	Hemizygous (males) or heterozygous (females) mutations	Hemizygous: 100%; Heterozygous: 25%
Autosomal recessive Alport syndrome	*COL4A4* or *COL4A3* /2q36-37	α4 and α3 chains of type IV collagen	Biallelic (homozygous or compound heterozygous) mutations	100%
Autosomal dominant Alport syndrome	*COL4A4* or/*COL4A3* 2q36-37	α4 and α3 chains of type IV collagen	Heterozygous mutations in the α4 or α 3 chains	20% in patients with risk factors for progression
Digenic Alport syndrome	Two of the *COL4A3-5* genes	Two of the α3-5 chains		

Males harbor hemizygous mutations whereas females carry heterozygous mutations. The *COL4A4* and *COL4A3* genes encode respectively the α4 and α3 chains of type IV collagen and are located on chromosome locus 2q36-37. Mutations in these genes cause autosomal Alport syndrome. Biallelic (homozygous or compound heterozygous) mutations in either one of them result in autosomal recessive Alport syndrome whereas autosomal dominant Alport syndrome is due to heterozygous mutations in either the *COL4A4* or *COL4A3* genes. Mutations in two of the *COL4A5*, *COL4A4*, or *COL4A3* genes cause digenic Alport syndrome[90-94].

Mutations in type IV collagen are highly prevalent. Genome-wide association studies show that 1 in 600 subjects from the Icelandic population carry a variant in the *COL4A3* gene associated with hematuria and albuminuria. In the UK population, the *COL4A4* variant rs35138315 (Ser969X) has a carrier frequency of 0.13% and is also associated with hematuria and albuminuria[95]. Among 24 Greek families with familial microscopic hematuria, next generation sequencing identifies pathogenic mutations in the *COL4A3-5* genes in 17 (71%) of them[96]. Mutations in the *COL4A3-5* genes are also frequently found in patients with sporadic and familial FSGS[94,95,97-99]. Pathogenic variants in any of the *COL4A3-5* genes are found in up to 10% of patients with renal failure of unknown cause and in some families with IgA nephropathy[94]. Therefore, indications for screening for pathogenic variants in the *COL4A5*, *COL4A4*, or *COL4A3* genes have been extended beyond the classical Alport syndrome phenotype (hematuria, renal failure, family history of hematuria or renal failure) to include FSGS, persistent proteinuria, steroid-resistant nephrotic syndrome, familial IgA nephropathy, and ESKD without an obvious cause[94].

The phenotypical expression of mutations in type IV collagen (*COL4A3-5* genes) is heterogeneous. Patients with type IV collagen-related nephropathy may exhibit isolated microscopic hematuria, proteinuria, or kidney failure that evolves to ESKD. In addition, patients with type IV collagen mutations may experience extrarenal manifestations such as sensorineural hearing loss, lenticonus, and retinopathy[90-93,97]. In patients with mutations in the *COL4A5* gene (X-linked Alport syndrome), hemizygous males have a 100% risk of progression to ESKD while heterozygous females (formerly called carriers) have substantial risk associated with proteinuria, progressive renal disease, and sensorineural hearing loss. Their lifetime risk of progression to ESKD is approximately 25%. Patients with autosomal recessive Alport syndrome (due to biallelic mutations in *COL4A4* or *COL4A3* genes) have a 100% risk of ESKD. Patients with heterozygous mutations in *COL4A4* or *COL4A3* genes (autosomal dominant Alport syndrome) may be asymptomatic or may exhibit hematuria or proteinuria and include patients previously diagnosed with thin basement membrane nephropathy. Risk factors for progression to ESKD in these subjects include proteinuria, sensorineural deafness, family history of progression to ESKD and renal biopsy findings of FSGS or GBM thickening and disarray. The risk of ESKD is up to 20% among those with risk factors. Patients with heterozygous mutations in *COL4A4* or *COL4A3* genes without kidney manifestations (hematuria or proteinuria) generally have a good prognosis but should be screened in a yearly basis[93].

The kidney histological phenotype of mutations in type IV collagen is characterized by GBM alterations, effacement of podocyte foot processes, and FSGS. Light microscopy examination of kidney samples from patients with Alport syndrome may reveal normal glomeruli or only minor mesangial widening. Immunofluorescent staining generally renders negative or nonspecific results. Electron microscopy usually provides the diagnosis, revealing changes in the GBM that may include areas of thinning, thickening, lamellation, and splitting. Initially, the GBM exhibits segmental thinning followed by progressive thickening and disorganization. In addition, diffuse podocyte foot process effacement occurs very frequently[74,97,99-101]. Patients with pathogenic variants affecting the α chains of type IV collagen may display FSGS with or without GBM changes[91,97-99,102,103].

Type V collagen in the normal glomerulus: In normal human glomeruli, type V collagen shows a distribution similar to type IV, being detectable in the GBM and the mesangium[38-40].

Type VI collagen in the normal glomerulus: In human adult glomerular tissue, mass spectrometry quantitative analyses show that type VI collagen is highly abundant[27]. In normal kidney samples, immunogold electron microscopy and immunohistochemical analyses show that the glomerular distribution of type VI collagen is comparable to that of the α1 chain of type IV collagen, namely along the mesangial matrix and the endothelial aspect of the GBM mainly[27,31,32,38,40].

Other types of collagen (type XV, type XVII and type XVIII collagen) in the normal glomerulus: Type XV collagen (α1 chain) has been found among ECM proteins in the glomerular proteome although its biological significance is uncertain[27].

Type XVII collagen is a transmembrane molecule involved in epithelial adhesion that has been identified as an autoantigen in bullous pemphigoid, a blistering skin disease of autoimmune origin. The association of bullous pemphigoid and a glomerular disease with characteristics of anti-GBM disease and membranous nephropathy has been reported in a 75-year-old man that also had circulating IgG against BP180, the 180-kDa bullous pemphigoid antigen (type XVII collagen). The kidney biopsy exhibited endocapillary inflammation without crescents. Direct immunofluorescence showed strong IgG and C3 staining in a combined granular and linear pattern along the GBM. Electron microscopy revealed subepithelial deposits[104]. In a kidney biopsy sample collected from a 4-year-old girl with hematuria, immunoelectron microscopy reveals that type XVII collagen is expressed in the foot processes of podocytes. In addition, type XVII collagen can be seen in the adjacent lamina rara externa of the GBM[105].

Type XVIII collagen has been identified among the ECM proteins in the glomerular proteome, being present in the GBM and the mesangium. Its expression pattern is similar to that of the α1 and α2 chains of type IV collagen[27,106].

Other components and factors that may modulate the normal composition of the glomerular ECM

The tubulointerstitial nephritis antigen-like-1 (TINAGL1) is highly abundant in normal glomerular ECM, being predominantly localized to the mesangial matrix. TINAGL1 is a glycoprotein structurally related to the tubulointerstitial nephritis antigen, a protein of the tubular basement membrane that is the antigenic target in autoimmune anti-tubular basement membrane disease. Nephronectin, vitronectin, fibulin-1, and fibrillin-1 have been identified as components of glomerular proteome using mass spectrometry. Nephronectin is present both in the GBM and mesangial matrix while vitronectin is localized in the mesangial matrix alone[27].

Matrix metalloproteinases and their inhibitors: Matrix metalloproteinases (MMPs) and their inhibitors are present in the ECM, but the particular isoforms distributed to the human kidney and their specific pathophysiologic role remain largely unknown. Disruption of the balance between MMPs and their inhibitors in the extracellular space has been implicated in the development of kidney fibrosis. Plasma concentration of MMPs and their inhibitors have been correlated with insulin resistance and kidney disease in clinical studies, suggesting that the composition of the ECM is altered in these conditions[107-109]. In the Renal Iohexol Clearance Survey, higher MMP-7 Levels were independently associated with increased risk of accelerated glomerular filtration rate (GFR) decline and incident chronic kidney disease among 1324 adults from the general population free of baseline diabetes, kidney disease or cardiovascular disease, over a median observation period of 5.6 years. In contrast, MMP2 and tissue inhibitor of metalloproteinase-1 (TIMP-1) showed no association with kidney disease[107]. Patients with insulin resistance display increased plasma TIMP-1 Level compared with healthy subjects. Accordingly, elevated plasma TIMP-1 concentration may be a marker of interstitial fibrosis due to excessive collagen deposition[108,109].

In vitro studies show that the expression of MMPs in the kidney ECM may be regulated at least in part by growth factors and ECM components[110,111]. In human kidney tubular cells, transforming growth factor-β1 (TGF-β1) induces MMP-2 expression *via* up-regulation of integrin-linked kinase[110], while elevated glucose concentration decreases MMP-9 and MMP-2 expression and increases TIMP-1 expression[112]. In cultured human glomerular epithelial cells, the expression of MMP-2 and MMP-9 is down-regulated by the presence of the α3 chain of type IV collagen[111], while high glucose concentration reduces MMP-2 expression and up-regulates TIMP-2[113].

Growth factors: The ECM composition is modulated by growth factors such as TGF-β1, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). TGF-β1 and PDGFs may promote ECM fibrosis in the kidney at least in part *via* integrins and integrin-associated proteins[114,115]. *In vitro* investigations using human mesangial cells show that TGF-β1 induces mesangial matrix expansion [116]. Accordingly, up-regulation of TGF-β1 is observed in the areas of interstitial and fibrosis in human fibrotic kidneys, compared with control kidneys[117]. Likewise, the expression of TGF-β1 and type IV collagen is increased in kidney allografts with interstitial fibrosis compared to normal kidney tissue [118]. VEGF and its receptors are expressed in normal human kidney, particularly in podocytes and mesangial cells. In normal podocytes, transmission electron microscopy examination reveals that VEGF may be detected in the intracellular compartment (36%) and associated with the cell membrane (63%) [119]. *In vitro* studies show that VEGF induces a proliferative effect on human mesangial cells[120,121].

The role of VEGF in glomerular pathophysiology is largely unknown, but neutralizing VEGF activity may increase the risk of kidney disease, as bevacizumab (a monoclonal antibody against human VEGF-A) therapy has been associated with elevated risk of proteinuria and hypertension among cancer patients in a systematic review and meta-analysis of clinical trials[122]. Rapamycin therapy has been associated with reduced VEGF expression in the human kidney that might contribute to explain the renal side-effects of this drug[123].

Integrins and integrin-associated proteins: Growth factors may interact with integrins to initiate signaling cascades. Integrins are plasma membrane proteins that link structurally and functionally the cell cytoskeleton with the extracellular space (Figure 3). Inside the cell, the cytoplasmic domain of integrins connects with the cytoskeleton *via* integrin-associated proteins, including integrin-linked kinase, particularly interesting new cysteine-histidine-rich protein (PINCH1), parvin proteins, and calponin homology domain-containing integrin-linked kinase-binding protein (CH-ILKBP)[114,124-128]. PINCH1 is an adaptor protein that comprises five LIM domains and interacts with integrin-linked kinase[129]. The parvins are partner proteins to integrin-linked kinase and PINCH1[130]. CH-ILKBP interacts with integrin-linked kinase, PINCH1, and the cytoskeleton. The interaction with integrin-linked kinase mediates the plasma membrane localization of CH-ILKBP. Northern blot analyses show widespread CH-ILKBP expression in human tissues, particularly in the heart, skeletal muscle, and kidney[131]. *In vitro* studies using human cell lines (HeLa cells) show that depletion of CH-ILKBP prevents the membrane translocation and the phosphorylation of protein kinase B (AKT), suggesting that CH-ILKBP facilitates the activation of this kinase in response to extracellular signals[132]. Integrins and integrin-associated proteins convey cues from growth factors and ECM components to intracellular pathways, although specific signaling cascades are not fully elucidated in humans[110,117,118]. Integrin signaling *via* integrin-associated proteins has been implicated in the regulation of ECM deposition and may be involved in the development of kidney fibrosis, both in native kidneys and kidney allografts, although underlying mechanisms remain largely unsolved[118]. An up-regulation of β1 integrin and integrin-linked kinase has been observed in areas of interstitial fibrosis in human fibrotic kidneys, compared with control kidneys[117]. *In vitro* experiments using cultured human proximal tubular cells reveal that overexpression of integrin-linked kinase and PINCH1 increases fibronectin-1 expression and its extracellular assembly, whereas PINCH1 knockdown reduces TGFβ1-mediated fibronectin-1 expression[110,124]. *In vitro* studies show that α3β1 integrin largely mediates the adhesion of human glomerular epithelial cells to type IV collagen[133]. Glucose concentration in the medium may alter integrin expression and the binding to type IV collagen in human glomerular epithelial cells[113], and human proximal tubular epithelial cells[112].

COMPOSITION OF THE GLOMERULAR ECM IN DKD

Diabetes is associated with a profound alteration in the composition of extracellular tissues throughout the body, including the kidney and the blood vessels. Patients with diabetes demonstrate increased interstitial collagen production and deposition that leads to fibrosis. Alström syndrome is an autosomal recessive disease due to mutations in the ALMS1 protein, characterized by the presence of early childhood insulin resistance. Like diabetes, patients with Alström syndrome typically show systemic fibrosis of extracellular tissues[5,36,134-136]. In patients with DKD, the amount and biochemical composition of the GBM and mesangial matrix are markedly anomalous. The global amount of glomerular ECM is increased, the level of heparan sulfate proteoglycans is reduced, and the collagen content is augmented compared to normal kidneys (Table 4)[36,134]. Furthermore, the abnormal composition of the glomerular ECM becomes more pronounced with the progression of DKD. Advanced sclerotic lesions show increased type III collagen and reduced amount of heparan sulfate proteoglycan and fibronectin-1 compared to earlier stages of DKD (Figure 4)[39,137,138].

Glomerular glycosaminoglycans, proteoglycans, and sialic acid in DKD

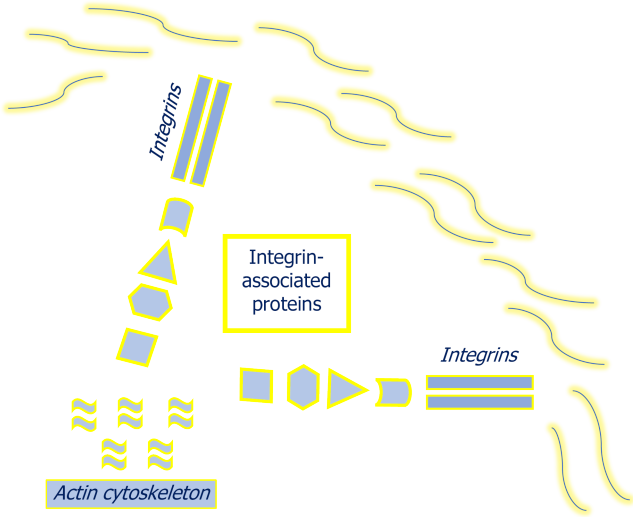
In patients with diabetes, the content of heparan sulfate in the glomerular ECM is prominently reduced while the global amount of extracellular tissue is increased. A quantitative assessment conducted by immunochemical procedures reveals that the abundance of heparan sulfate proteoglycan in the GBM of patients with diabetes is 30% lower than that of control subjects. The decrease in glomerular heparan sulfate has been also observed in other diseases, such as C3 glomerulopathy, membranous nephropathy, minimal change disease, and lupus nephritis[33,36,37,139].

The reduction in glomerular heparan sulfate proteoglycan associated with DKD starts to occur early and becomes more severe with the advance of the disorder. In patients with mild diffuse glomerulosclerosis, the staining pattern of heparan sulfate proteoglycan is reduced in the thickened mesangial matrix while in more pronounced diffuse glomerulosclerosis and mesangial nodules the enlarged matrix lacks heparan sulfate proteoglycan completely[33,39]. In contrast, the amount of hyaluronic acid is increased in the glomerular ECM of patients with DKD compared to control subjects[140]. As mentioned, heparan sulfate is a major ligand for factor H, an inhibitor of the alternative pathway of

Table 4 Different composition of glomerular extracellular matrix (glomerular basement membrane and mesangial matrix) in normal subjects and patients with diabetes

	Normal glomeruli	*Diabetic kidney disease*
Heparan sulfate proteoglycans	GBM and mesangial matrix	Decreased amount
Laminin	Predominantly in the GBM	Inconsistent
Fibronectin-1	Mainly in the mesangial matrix	It varies according to DKD stage
Type I collagen	Inconsistent	No detectable
Type III collagen	Absent	Abundant
Type IV collagen	Abundant in the GBM	Reduced GBM amount
Type V collagen	Similar to type IV collagen	Increased mesangial amount
Type VI collagen	GBM and mesangial matrix	Increased mesangial amount

GBM: Glomerular basement membrane; DKD: Diabetic kidney disease.

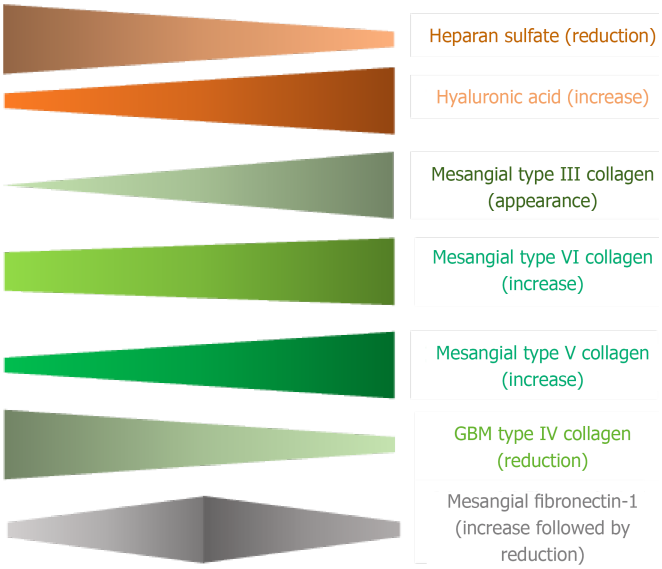


DOI: 10.4239/wjd.v13.i7.498

Figure 3 Integrins and integrin-associated proteins.

complement on "self" biological surfaces. Loss of heparan sulfate (or altered sulfation pattern) may result in reduced factor H attachment to "self" structures, subsequent activation of the alternative pathway and complement-mediated injury, like occurs in the presence of mutated factor H (C3 glomerulopathy). Complement activation *via* the alternative pathway may contribute to the progression of renal and vascular complications in human diabetes. In patients with biopsy-proven DKD, a higher level of factor H in the urine has been independently associated with worse kidney outcomes, including onset of ESKD and faster kidney function decline, compared to control subjects[141]. Further, clinical studies have shown an association between single nucleotide polymorphisms in factor H and adverse clinical outcomes in different population groups of non-diabetic and diabetic patients[142,143]. In African American patients, genetic changes in factor H gene, such as the intronic variant rs379489, have been associated with ESKD in both non-diabetic and type 2 diabetes (T2D), compared to controls[142]. In 1158 T2D patients prospectively followed in the randomized controlled trial Bergamo Nephrologic Complications of T2D (BENEDICT), the single nucleotide polymorphism in the factor H gene c.2808G>T (p.Glu936Asp) is independently associated with increased risk of microalbuminuria and cardiovascular complications (Asp/Asp homozygotes, recessive model). T2D patients Asp/Asp homozygotes are at increased risk of microalbuminuria and cardiovascular events compared to carriers of one or two wild type Glu alleles[143].

Among patients with diabetes, the reported amount of glomerular sialic acid has been inconsistent. A decline in the content of sialic acid has been detected in the glomerular ECM of patients with diabetes, compared to normal kidney samples[68,144]. However, an increased expression of sialic acid on podocytes has been observed in patients with DKD and other kidney diseases without differences among them[44].



DOI: 10.4239/wjd.v13.i7.498

Figure 4 Schematic variation in some components of the glomerular extracellular matrix according to the progression of diabetic kidney disease. GBM: Glomerular basement membrane.

A study that applied weighted gene co-expression network analysis to 179 human glomeruli reveals that two small leucine-rich proteoglycans (lumican and fibromodulin) are more abundant in the ECM of patients with DKD compared to controls and other glomerular diseases, such as IgA nephropathy or membranous nephropathy. Further, the expression level of lumican and fibromodulin is negatively correlated with kidney function. The specificity of lumican and fibromodulin in kidney samples from patients with DKD in comparison to normal specimens and patients with other glomerular diseases suggests that these ECM components may become potential diagnostic biomarkers for DKD[14].

Glomerular laminin in DKD

The laminin content of the glomerular ECM in patients with diabetes has been barely reported and the values are variable[37,39]. In human kidneys obtained at autopsy, there is a marked reduction in laminin content in the diabetic GBM compared to non-diabetic control subjects. Radioimmunoassays indicated that GBM from patients with diabetes contains average values of laminin that were 60% of control subjects[37]. However, immunohistochemical studies show an increased glomerular deposition of laminin in kidney biopsy samples from type 1 diabetes (T1D) and T2D patients with diffuse and nodular glomerulosclerosis[39].

Glomerular fibronectin-1 in DKD

Immunohistochemical studies reveal that the amount of mesangial fibronectin-1 is abnormal in patients with diabetes and varies with the advance of DKD. Antibodies to fibronectin-1 normally stain the mesangium and the subendothelial aspect of the GBM. Early lesions of mesangial expansion are associated with increased staining for fibronectin-1. However, a marked diminution in fluorescent intensity for fibronectin-1 is documented in more advanced mesangial enlargement (nodular lesions). Compared with normal tissues and early lesions of DKD, the progression of the disease is associated with a noticeable reduction in the amount of glomerular fibronectin-1[39,40,137]. The increase in mesangial fibronectin-1 that occurs in the early stage of DKD also takes place in other glomerular diseases characterized by mesangial expansion, such as mesangiocapillary glomerulonephritis[59,60]. An up-regulation of fibronectin-1 expression in the glomeruli has been also observed in patients with hypertension compared to normal kidneys[4]. Likewise, the amount of fibronectin-1 in vascular tissue is increased in patients with diabetes before the development of atherosclerosis lesions[59,145]. The content of fibronectin-1 in the intima-media of normal aorta specimens is more elevated in patients with diabetes (T1D and T2D) compared to control subjects, suggesting that diabetic patients develop structural alterations in the connective tissue of their arteries before the appearance of vascular disease [145]. In patients with DKD, the thickened capillary walls also contain a markedly elevated amount of fibronectin-1[59].

Glomerular collagen in DKD

Earlier studies found elevated glomerular ECM levels of glycine, hydroxyproline, hydroxylysine, and hexoses in patients with diabetes compared to normal kidney samples, suggesting an increase in the amount of collagen in the glomerular ECM from diabetic patients[30,36,66-68]. Radioimmunoassays

confirmed collagen enrichment in the glomerular ECM of patients with diabetes[37]. Accordingly, electron microscopy examination shows accumulation of collagen fibrils in the mesangium of patients with DKD[146].

Glomerular type I collagen in DKD: No glomerular type I collagen has been detected in DKD at any stage of the disorder[33,39,40,137,147].

Glomerular type III collagen in DKD: Unlike normal glomeruli, type III collagen is identified in the mesangium of patients with DKD and its amount increases gradually with the progression of the disease. Early DKD lesions (diffuse glomerulosclerosis) show positive staining for type III collagen that increases in more advanced mesangial nodular lesions. In the late stage of global sclerosis, type III collagen is diffusely present in the sclerotic mesangial matrix. Therefore, *de novo* synthesis of type III collagen in glomeruli occurs in patients with DKD[33,39,40,70,71]. A patient with T1D and collagenofibrotic glomerulopathy has been reported[148].

Glomerular type IV collagen in DKD: In patients with diabetes, immunohistochemical estimates of type IV collagen in the GBM reveal reduced staining compared to normal tissue. Accordingly, the density of gold particles for type IV collagen is decreased in the GBM of T1D patients on quantitative immunogold electron microscopy examination. Like in normal subjects, the labeling of antibody against type IV collagen in the GBM is concentrated in the endothelial zone and decreases towards the epithelial aspect of the GMB in diabetic patients[33,89].

In the mesangial matrix, immunohistochemical studies show that the amount of type IV collagen changes according to the stage of DKD. In earlier lesions of diffuse glomerulosclerosis, mesangial staining for type IV collagen is increased while more advanced nodular glomerulosclerosis showed marked reduction in the mesangial staining for type IV collagen, suggesting that type IV collagen is progressively substituted for other collagen types such as type VI and type III during the transition from diffuse to nodular glomerulosclerosis[39,40,89]. However, an elevated mesangial staining for type IV collagen has been observed in specific nodular lesions, called non-mesangiolytic nodules, compared to normal kidney[33,71]. The amount of type IV collagen in nodular lesions may depend on the type of the lesion, mesangiolytic or non-mesangiolytic. In a study aimed to investigate collagen staining of mesangial nodules from 67 patients with DKD, type IV collagen staining was only robust in nodular lesions with strong PAS/periodic acid methenamine silver (PAMS) staining (non-mesangiolytic nodular lesions). In contrast, nodular lesions with faint PAS/PAMS staining (mesangiolytic nodular lesions) did not show type IV collagen. The amount of type IV collagen correlates with the PAS and PAMS staining pattern. Non-mesangiolytic nodules (with prominent PAS/PAMS staining) are strongly positive for type IV collagen whereas mesangiolytic nodules (with weak or negative PAS/PAMS staining) show weak or negative staining for type IV collagen[147]. Immunofluorescence studies performed in 918 kidney biopsy samples from patients with diabetes (T1D and T2D) show accumulation of α3 and α5 chains of type IV collagen in diffuse mesangial sclerosis while minimal amounts of these α3 and α5 chains were seen within the mesangium of control subjects[43].

In patients with diabetes, two large clinical investigations with different population groups (African American and European descent subjects) have shown that genetic variants in the gene that codes the α3 chain of type IV collagen (*COL4A3*) may modulate susceptibility to DKD and ESKD[149]. In 4885 African American patients with T2D, an association between the genetic variant R408H (rs34505188) in *COL4A3* and ESKD has been observed, suggesting that genetic changes in the *COL4A3* locus may contribute to ESKD susceptibility in patients with diabetes[149]. In 19406 T1D patients of European descent from 17 cohorts, a genome-wide association meta-analysis reveals that a single nucleotide polymorphism in the *COL4A3* gene is associated with protection from DKD (proteinuria and ESKD)[150].

Glomerular type V collagen in DKD: Immunohistochemical studies have documented an enrichment in mesangial type V collagen in diffuse glomerulosclerosis and nodular lesions in patients with DKD compared to control subjects. Increased staining for type V collagen is observed in advanced mesangial disease, compared to normal tissues and early mesangial disease.

Staining for type V collagen was strongly positive in all nodular lesions, mesangiolytic and non-mesangiolytic[39,40,137,147].

Glomerular type VI collagen in DKD: In patients with DKD, the amount of mesangial type VI collagen is elevated. Quantitation by radioimmunoassay reveals that the level of type VI collagen is 2.8-fold higher in the diabetic preparations compared to control subjects. Furthermore, the amount of mesangial type VI collagen increases with the progression of DKD. In earlier lesions of diffuse glomerulosclerosis, the contribution of type VI collagen deposition to the overall matrix expansion is minor. However, type VI collagen is a major component in the expanded mesangial matrix of nodular glomerulosclerosis. A marked increase in type VI collagen deposition is observed in nodular lesions where the strong positivity for type VI collagen is evenly distributed throughout the entire nodules[31,39,40,147].

Other factors that contribute to modulate the composition of kidney ECM in DKD

As kidney ECM remodeling is profoundly altered in patients with DKD, the expression of MMPs, TIMPs, integrins, integrin-associated proteins, and signaling pathways from growth factors have been reported abnormal among these patients. In addition, the nuclear factor-kappa-B (NF-κB) family of transcription factors and advanced glycation end-products (AGEs) have been proposed as potential contributors to the ECM disturbance present in patients with DKD.

MMPs and their inhibitors in patients with DKD: The expression of MMPs and their inhibitors is altered in patients with DKD. Clinical studies have suggested that these ECM components might be useful to evaluate the risk for cardiovascular disease, kidney disease, and all-cause mortality among patients with diabetes. In a cross-sectional study, T1D patients with cardiovascular disease showed higher levels of TIMP-1 compared to T1D patients without cardiovascular disease[151]. In a prospective study that followed 337 T1D patients for a median period of 12.3 years, elevated MMP-2 plasma levels were associated with higher incidence of cardiovascular events, but this relationship was attenuated after adjustment for estimated GFR, suggesting that kidney function may mediate the association[152]. In a cross-sectional pooled analysis of three groups of T1D patients, circulating MMP-1, MMP-2, and MMP-3 Levels were associated with arterial stiffening independent of confounding factors while no association with TIMPs was observed[153]. In a case-control study that evaluated 120 control women and 120 women with a history of gestational diabetes 3.7 years after delivery, both serum TIMP-1 Levels and arterial stiffness were higher in subjects with previous gestational diabetes compared to control individuals[154]. In T1D patients, MMPs and their inhibitors have been associated with albuminuria in a cross-sectional study[151], and with kidney function decline in a prospective study[152]. In a prospective observational cohort study, urinary excretion of MMP-7 was independently associated with higher mortality rate over a median follow-up period of 3.0 years, in T2D patients with DKD. In contrast, no association between serum MMP-7 Level and mortality was observed[155]. In T1D patients, the association of MMP-1, MMP-2 and MMP-3 with all-cause mortality was attenuated after adjustment for estimated GFR, suggesting that the known association between kidney function and mortality may mediate the relation between MMPs and death[152].

However, MMPs expression in glomeruli may be altered in other glomerular diseases, such as IgA nephropathy, which is associated with extensive changes of the glomerular ECM proteome, including higher abundance of MMP-9, MMP-2, α1 chain of type IV collagen, fibronectin, and β1-laminin[156].

Growth factors (TGF-β, PDGF, and VEGF) in patients with DKD: In T2D patients with albuminuria, serum TGF-β1 Level is higher compared to healthy controls and T2D patients with normal urinary albumin excretion rate, suggesting that serum TGF-β1 might be used to evaluate progression of DKD [157,158]. Several meta-analyses indicate a potential value of serum TGF-β1 Levels to evaluate the risk of DKD and the advance of the disease[159-161]. However, the administration of a neutralizing monoclonal antibody against TGF-β1 to T1D and T2D patients with DKD failed to slow progression of the disease compared to placebo in a randomized controlled clinical trial, suggesting that TGF-β1 is not a major determinant of kidney function decline in patients with diabetes[162].

The glomerular expression of PDGF-B and its receptor (PDGFR-β) is higher in T2D patients with DKD compared to normal kidneys, particularly in samples with mild mesangial expansion. In contrast, the expression of PDGF-A and its receptor (PDGFR-α) is comparable in normal kidneys and patients with DKD[163].

In patients with DKD, VEGF-A expression is lower compared to normal kidney tissue[164]. VEGF signaling has been reported to be differentially regulated in patients with DKD in a study that examined gene-expression changes in human DKD[165]. However, in a prospective study that recruited 155 T1D patients with proteinuria, plasma VEGF failed to predict kidney function decline over 3-year follow-up [166].

Integrins and integrin-associated proteins in patients with DKD: Glomerular integrin and integrin-linked kinase signaling pathways have been found differentially regulated in patients with DKD[165]. In addition, the expression of integrin-linked kinase in the mesangium is increased in kidney specimens from patients with diabetes and diffuse mesangial expansion, compared to control samples. In contrast, integrin-linked kinase level is reduced in glomeruli with advanced nodular sclerosis and global sclerosis, suggesting that integrin-linked kinase expression increases during early stages of DKD[114].

NF-κB in patients with DKD: NF-κB is a transcription factor that regulates the expression of several genes. NF-κB-inducing kinase activates the NF-κB signaling pathway. Dysregulation of NF-κB signaling has been implicated in DKD, but its role remains uncertain. In vitro studies using human proximal tubular epithelial cells (HK-2 cells) suggest a role for NF-κB pathway in modulating diabetes-induced disease in renal tubular epithelium[167,168].

Advanced glycation products in patients with DKD: AGEs are molecules that result from nonenzymatic glycation of proteins and lipids. They may bind to cell surface receptors (RAGEs). AGEs have been hypothesized to be involved in the development of human DKD, but their participation remain undefined. In kidney biopsies from patients with DKD, AGEs are detected in the expanded

mesangial matrix while they are not identified in control samples[169,170]. In addition, AGEs are identified in areas of glomerulosclerosis and arteriosclerosis in other diseases, such as FSGS, hypertensive nephrosclerosis, and lupus nephritis[169]. RAGE expression was detected in mesangial cells and glomerular epithelial cells, in both patients with DKD and control subjects[170]. In a cross-sectional study, the level of AGEs was positively associated with serum concentration of MMP-2, MMP3, and TIMP-1 while an inverse association with MMP-9 was observed in T1D patients[171].

CONCLUSION

Understanding mechanisms that regulate glomerular ECM injury and repair may contribute to develop therapeutic strategies for DKD and other kidney diseases. During adult life, mesangial cells produce mesangial matrix. The turnover of the GBM present at birth is unknown. Podocyte foot processes surround and attach entirely the GBM. Adult podocytes may sustain hypertrophy following the loss of adjacent cells to prevent bared GBM areas that compromise the filtration barrier. Glycosaminoglycans, such as heparan sulfate and hyaluronic acid, are major constituents of the glomerular ECM. The specific pattern of sulfation of glycosaminoglycans allows the identification of these molecules as "self" by complement components and avoid complement-mediated self-damage. Sialic acid is also present in glomerular ECM and may serve to a similar function. Fibronectin-1 is important for the normal deposition of other ECM components, such as collagen. Type IV, V, and VI collagens are predominant types of collagen normally present in the glomerular ECM while type III collagen appears in diseased states, such as diabetes and glomerulosclerosis. The composition and arrangement of the glomerular ECM is profoundly altered in patients with diabetes. The global quantity of glomerular ECM is increased while the amount of sulfated proteoglycans is reduced and hyaluronic acid is augmented, compared to control tissue. Fibronectin-1 is increased in early lesions of mesangial expansion. Likewise, the amount of fibronectin-1 in capillary walls and aorta is increased before the development of vascular disease in patients with diabetes. Mesangial type III, type V, and type VI collagen amount is elevated in patients with DKD and increases progressively with the advance of the disease. Genetic variants in the gene that codes the α3 chain of type IV collagen (*COL4A3*) may modulate susceptibility to DKD and ESKD.

ACKNOWLEDGEMENTS

We are very grateful to Ms. Gema Souto, MHS for her help and support during the writing of this manuscript.

FOOTNOTES

Author contributions: Adeva-Andany MM conceived and designed the review, performed the literature search and wrote the original draft of the manuscript; Carneiro-Freire N contributed to the writing and revision of intellectual content; both authors participated in the final version of the manuscript.

Conflict-of-interest statement: The authors declare that there is no conflict of interest.

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 521-531

DOI: 10.4239/wjd.v13.i7.521

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Association between urinary concentrations of bisphenol A substitutes and diabetes in adults

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: He Z, China; Rojas A, Chile

Received: March 11, 2022
Peer-review started: March 11, 2022
First decision: April 17, 2022
Revised: April 26, 2022
Accepted: June 22, 2022
Article in press: June 22, 2022
Published online: July 15, 2022

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Abstract

BACKGROUND

Due to new restrictions on the use of bisphenol A (BPA), industries are beginning to replace it with derived molecules such as bisphenol S and F (BPS and BPF). There is extensive evidence in the academic literature on the potential health effects of BPA, which is known to be a diabetogenic molecule. However, there are few publications related to new compounds derived from BPA.

AIM

To perform an epidemiological study of urinary BPS and BPF in the American National Health and Nutrition Examination Survey (NHANES) cohort, and analyze their possible relationship with diabetes mellitus.

METHODS

NHANES datasets from 2013 to 2016 were used due to the urinary BPF and BPS availability. Data from 3658 adults were analyzed to perform regression analysis exploring the possible relationship between BPA-derived compounds and diabetes.

RESULTS

Descriptive statistics, linear regression modeling, and logistic regression analysis revealed a significant relationship between urinary BPS, but not BPF, and diabetes

risk. Additionally, a relationship was observed between both compounds and hypertension and a slight relationship between BPF and dyslipidemia.

CONCLUSION

In the present study, a strong relationship between urinary BPS, not BPF, and diabetes risk has been determined. BPA substitute molecules do not exempt the population from potential health risks.

Key Words: Bisphenol S; Bisphenol F; Diabetes mellitus; National Health and Nutrition Examination Survey; Urine

Core Tip: Molecules derived from bisphenol A (increasing use in the plastic industry and the production of heat-sensitive tickets) could be related to pathologies such as diabetes (bisphenol S) and hypertension (bisphenol S and F).

Citation: Moreno-Gómez-Toledano R, Vélez-Vélez E, Arenas MI, Saura M, Bosch RJ. Association between urinary concentrations of bisphenol A substitutes and diabetes in adults. *World J Diabetes* 2022; 13(7): 521-531
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/521.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.521

INTRODUCTION

In the last decades, the demand and production of plastic polymers have increased substantially. Both its production and recycling involve the release of pollutants, xenobiotic compounds that should not be found in the air, rivers, or the human population[1,2]. One of the most important, due to its wide distribution and variety of biological effects, is bisphenol A (BPA)[3-6]. The European Chemicals Agency has recently included BPA within the Candidate List of substances of very high concern due to its properties as an endocrine disruptor and its potentially harmful effect on reproduction[7]. Furthermore, the European Union has restricted the use of this substance in thermal paper due to its potential danger to the health of exposed workers[8].

Therefore, the need to seek alternatives to BPA is a fact of vital importance for modern industry. Currently, two known compounds are bisphenol S (BPS) and bisphenol F (BPF), which can already be found in BPA-free packaging and thermal tickets regulated by European legislation[9,10]. The use of these derivatives does not imply a reduction in possible adverse effects *per se*; it only indicates the use of new materials whose safety has not yet been tested. Comparative studies between BPA substitutes have shown that both BPF and BPS are as hormonally active as BPA[9], so they could also be included in the category of endocrine disruptors.

Diabetes mellitus (DM) and its associated complications are a medical catastrophe of global dimensions[11]. The number of people affected has risen from 108 million in 1980[12] to almost 500 million today[13]. The latest estimates suggest that it could rise to 578 million in 2030 and 700 million in 2045[13]. Risk causes for the disease include a combination of genetic and metabolic factors. There are non-modifiable factors, such as ethnicity or age, and modifying factors, such as diet, obesity, or smoking [14]. The multiplicity of factors that influence the development of the disease implies that environmental pollutants could also affect it. There is evidence that BPA exposure correlates with the risk of developing DM[15].

However, new alternative compounds to BPA have only been used in modern industry for a short time. For this reason, few academic publications study its possible relationship with diabetes. The first pieces of evidence have been detected in a cellular experimental model[16], in males (but not females) of a murine experimental model[17], and in human cohorts from China[18,19] and France[20]. However, it has not yet been studied in one of the world's largest urinary bisphenol cohorts, the American National Health and Nutrition Examination Survey (NHANES). Studies in this cohort have demonstrated the presence of BPF and BPS in urine, observing positive and statistically significant relationships with disorders such as asthma[21], obesity[22-24], or depression[25]. Obesity is closely related to diabetes[26-28], so studying its relationship with new environmental pollutants is coherent and necessary. The present work aimed to correlate, for the first time in the NHANES cohort, diabetes with the urinary concentration of BPA substitutes using regression models.

MATERIALS AND METHODS

NHANES 2013-2016 population

The NHANES datasets from 2013 to 2016 were used in the present statistical model due to the urinary BPF and BPS availability. In the first phase, the data of all the study participants were extracted through the official website of the Centers for Disease Control and Prevention[29] (accessed December 01, 2021), obtaining 20146 individuals. Subsequently, the individuals with available BPS and BPF were selected, obtaining 5333 subjects, of which 3699 were adults (over 18 years of age). Data from 3658 patients could be used for regression models (complete data). Subsequently, two classifications were made: Group 1 was performed with individuals with and without diabetes. Groups 2 and 3 were performed by analyzing the individuals based on the concentration of urinary phenols (BPS for group 2 and BPF for group 3).

All individuals whose doctor had diagnosed them with diabetes, those taking blood glucose medication, and individuals with a fasting glucose value ≥ 126 mg/dL or hemoglobin A1c ≥ 6.5% were included in the diabetic group. The individuals classified according to the concentration of phenols were divided into four quartiles for BPS and BPF (Q1-Q4). BPS and BPF values were corrected for urinary creatinine to normalize variations due to hydration or glomerular filtration capacity[30].

Binary logistic regression models were corrected for factors such as age, sex, body mass index (BMI), smoking, hypertension, or dyslipidemia. All those patients diagnosed by their doctor, those with medication for hypertension, and individuals with systolic pressure ≥ 140 mmHg or systolic ≥ 90 mmHg were considered hypertensive. The patients with dyslipidemia were those with diagnosed cholesterol disorders, with prescribed medication or fasting total cholesterol ≥ 240 mg/dL[31]. For smoking, all individuals who answered affirmatively to the question "have you smoked more than 100 cigarettes in your life?" or individuals with a serum cotinine value greater than 10 mg/dL[32] were included.

Statistical analysis

The IBM SPSS Statistics 27 program was used for the statistical analyses to carry out linear regression and logistic regression analyses, and the GraphPad Prism 7.0 program was used for basic descriptive statistics and comparative analysis. In the comparative analysis of the diabetes subgroup, the Mann-Whitney test was used. In the case of classification based on the phenol quartile, the Kruskal-Wallis test was used. The linear regression analysis used the R-squared coefficient of determination to define the percentage of change in the dependent variable affected by the independent variable. The ANOVA test was used to validate the statistical significance of the coefficient. Finally, the β coefficients and their statistical significance were calculated.

Since the diabetes variable is dichotomous, a binary logistic regression model was used. BPF and BPS values were analyzed with the corresponding correction with urinary creatinine, using their logarithmic transformation to normalize the non-parametric distribution. Three different regression analyzes were performed for each parameter: (1) Individual; (2) Corrected for age, sex, and BMI; and (3) Corrected for the above parameters and smoking, hypertension, and dyslipidemia.

In the study of groups 2 and 3, a multinomial logistic regression model was used. As in the previous statistical model, age, sex, BMI, smoking, hypertension, and dyslipidemia were also included. In all cases, those results whose *P* value was less than 0.05 were interpreted as statistically significant.

RESULTS

General data

Descriptive statistical analyses showed, in addition to the expected differences related to blood glucose, interesting changes in BPS levels, significantly higher in diabetic patients. However, the BPF values did not show significant variations. In addition, diabetic patients had higher age, BMI, and systolic pressure and lower total cholesterol (Table 1).

Descriptive analyses of group 2 (distributed according to BPS quartile) showed that individuals with a higher concentration of BPS (Q4) had a significant increase in BMI, fasting glucose, and BPF than individuals with a lower level of BPS (Q1) (Table 2). In addition, the percentages showed a positive and dose-dependent relationship between the BPA quartile and the number of patients with diabetes, hypertension, and dyslipidemia. Interestingly, the percentage of men showed a negative trend with urinary BPS concentration, and a positive trend was observed between the percentage of individuals with diabetes, hypertension, or dyslipidemia, and urinary BPS concentration.

On the other hand, the descriptive analyses of group 3 (distributed according to the BPF quartile) showed significant age differences (in quartiles 2, 3, and 4), BMI (Q2 and Q3), and cotinine (the quartile 4 had a significantly higher concentration than the other three quartiles) (Table 3). In this group, no significant differences were observed in the parameters related to diabetes, but an interesting positive relationship was observed in the percentage of individuals with hypertension.

Table 1 Descriptive statistics of main variables analyzed in individuals of group 1 (diabetes)

	Non-diabetic	Diabetic
n	3017	641
Age	41.11 (40.48-41.75)	58.33 (57.15-59.53)[d]
Gender, % of men	46.6	51
BMI, kg/m^2	27.82 (27.6-28.04)	31.4 (30.86-31.94)[d]
Fasting glucose, mg/dL	98.08 (97.56-98.6)	149.6 (144.4-154.9)[d]
HbA1c, %	5.41 (5.40-5.43)	7.26 (7.14-7.39)[d]
Cotinine, serum, ng/mL	0.28 (0.24-0.31)	0.18 (0.13-0.24)[a]
Smoker, %	42.5	50.2
Systolic blood pressure, mmHg	121 (120.4-121.6)	130.2 (128.7-131.7)[d]
Diastolic blood pressure, mmHg	68.86 (68.42-69.31)	68.21 (67.17-69.26)
Hypertension, %	36.1	71.9
Dyslipidemia, %	35	66.6
Total cholesterol, mg/dL	187.4 (186-188.9)	180.7 (177.2-184.2)[c]
Bisphenol F, µg/g creatinine	0.41 (0.39-0.43)	0.43 (0.38-0.48)
Bisphenol S, µg/g creatinine	0.5 (0.48-0.52)	0.59 (0.53-0.64)[b]

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
[d]$P < 0.0001$.
The results are expressed as percentages (%) or as geometric mean (95%CI). BMI: Body mass index; HbA1c: Hemoglobin A1c.

Simple linear regression

Linear regression analyses were performed using fasting glucose and hemoglobin A1c (HbA1c) values to explore the relationship between diabetes and phenols. As shown in Table 4, the results were significant in the BPS group, while BPF did not show a statistical relationship with these parameters.

Binary logistic regression

The subsequent binomial logistic regression analysis performed on the dichotomous dependent variable diabetes confirmed the data observed in the linear regression (Table 5). Thus, it was observed that the urinary concentration of BPS, both individually and corrected for other factors, was an independent factor related to diabetes mellitus. However, this relationship could not be determined in the urinary concentration of BPF.

Multinomial logistic regression

This statistical analysis model showed a significant relationship between diabetes and BPS, but not BPF (Table 6). However, statistically significant data were only observed in the first two models (individual and corrected for sex, age, and BMI). Although it did not become significant when corrected for all the parameters, the resulting *P* value was 0.063.

Interestingly, in the BPS study, Q4 individuals showed a positive and significant relationship with gender, with an odds ratio (OR) (95%CI) of 1.94 (1.61-2.35) for women. An important relationship was also observed in the risk of suffering hypertension, with an OR of 1.26 (1.01-1.57). In the BPF study, the same significant relationship was observed in gender, with an OR of 2.13 (1.75-2.58) for women. Finally, a positive relationship was observed with smoking [OR of 1.78 (1.47-2.17)] and slightly negative with the BMI [0.98 (0.97-0.998)].

Complementary study of significant pathologies in regression models

Due to the results observed in the regression models and the trends observed in the descriptive statistics, a binomial logistic regression model was established, using hypertension or dyslipidemia as the dependent variable, in order to relate the risk of suffering from any of them depending on the concentration of urinary phenols. As shown in Table 7, urinary BPS is an independent factor related to hypertension. BPF, on the other hand, showed a statistically significant relationship when analyzed individually with both hypertension and dyslipidemia. This relationship held when correcting for age,

Table 2 Descriptive statistics of main variables analyzed in individuals of group 2 (bisphenol S)

BPS quartile	Q1	Q2	Q3	Q4
n	915	911	894	938
Age	42.88 (41.65-44.14)	42.42 (41.22-43.65)	44.5 (43.32-45.72)	45.8 (43.89-46.3)[d]
Gender, % of men	56.3	49.7	42.8	40.6
BMI, kg/m^2	27.76 (27.36-28.17)	28.41 (27.99-28.83)	28.94 (28.51-29.38)[c]	28.57 (28.15-29)[a]
Diabetes mellitus, %	15.3	16.6	18.1	20
Fasting glucose, mg/dL	103.7 (101.8-105.8)	104.5 (102.4-106.6)	108.2 (105.5-111)	108.8 (106.3-111.5)[a]
HbA1c, %	5.62 (5.57-5.68)	5.66 (5.61-5.72)	5.75 (5.69-5.81)	5.78 (5.71-5.85)
Smoker, %	43.6	44.8	42.4	44.5
Cotinine, serum, ng/mL	0.24 (0.19-0.32)	0.27 (0.21-0.35)	0.24 (0.18-0.31)	0.27 (0.21-0.35)
Hypertension, %	39	40.6	43.4	46.3
Systolic blood pressure, mmHg	120.6 (119.5-121.7)	123.1 (122-124.2)	122.5 (121.4-123.7)	123.9 (122.7-125.1)
Diastolic blood pressure, mmHg	68.31 (67.5-69.13)	69.36 (68.52-70.21)	69.18 (68.37-69.99)	68.18 (67.37-69)
Dyslipidemia, %	39.3	40.6	40.8	41.5
Total cholesterol, mg/dL	184.3 (131.6-187)	184.7 (182-187.3)	187.7 (184.9-190.5)	188.3 (185.7-190. 9)
Bisphenol F, μg/g creatinine	0.37 (0.34-0.41)	0.43 (0.39-0.47)[a]	0.41 (0.38-0.45)	0.45 (0.41-0.49)[b]

[a]$P < 0.05$, significant differences with respect to group Q1.
[b]$P < 0.01$, significant differences with respect to group Q1.
[c]$P < 0.001$, significant differences with respect to group Q1.
[d]$P < 0.05$, significant differences between Q2 and Q4.
The results are expressed as percentages (%) or as geometric mean (95%CI). BMI: Body mass index; HbA1c: Hemoglobin A1c; BPS: Bisphenol S; BPF: Bisphenol F.

sex, and BMI for hypertension, but not for dyslipidemia. Finally, no significant relationship was determined after correction for the rest of the parameters.

DISCUSSION

In the present work, it has been demonstrated, for the first time in the NHANES cohort, that BPS, but not BPF, is related to diabetes. The academic literature includes few publications that explore the BPS-diabetes or BPF-diabetes paradigm. There are only three relevant epidemiological studies; two studied type 2 diabetes[18,20], and the remaining investigated gestational diabetes[19].

Duan *et al*[18] considered that all individuals with fasting glucose ≥ 7.0 mmol/L or HbA1c ≥ 6.5% had type 2 diabetes mellitus. After performing the logistic regression analysis, they determined an OR (95%CI) of 1.73 (1.37-2.18) for the urinary BPS, analogous to the results observed in this study.

Rancière *et al*[20] conducted a longitudinal study analyzing the cases of type 2 diabetes developed over 9 years in the DESIR cohort. Due to the low rate of detection of urinary BPS (less than 15%), the statistical model was established comparing individuals with detectable levels of BPS with those in whom the compound had not been detected, obtaining a higher (significant) risk of developing type 2 diabetes in those individuals with detectable levels of BPS. The detection rate in the NHANES cohort was 57.1% and 88.4% for BPF and BPS, respectively[21]. The results also support and reaffirm those obtained in the present work, although the difference in the detection ratio is very striking. Völkel *et al*[33,34] exclusively quantified BPS-glucuronide, the main metabolized form, according to human pharmacokinetic models. In the case of the NHANES cohort, the total concentration of bisphenol was analyzed after previously deconjugating the metabolized forms (glucuronide and sulfate) with Helix pomatia enzymes.

Lastly, Zhang *et al*[19] analyzed BPS and BPF in a cohort of Chinese pregnant women to study their possible relationship with gestational diabetes mellitus. Interestingly, quantitative analyses detected BPS and BPF in most urine samples from pregnant women (greater than 90% in both cases). However, the regression models did not show significant relationships with either compound. They only determined a slight but significant increase in glucose related to urinary BPS concentration. Finally, when studying the relationship between blood glucose and urinary BPS according to fetal sex, they

Table 3 Descriptive statistics of main variables analyzed in individuals of group 3 (bisphenol F)

BPF quartile	Q1	Q2	Q3	Q4
n	912	912	922	912
Age	41.22 (40.04-42.42)	44.81 (43.59-46.06)[c]	45.22 (44.02-46.46)[d]	43.69 (42.5-44.92)[a]
Gender, % of men	58.2	44.5	43.7	43
BMI, kg/m^2	29.04 (28.61-29.48)	28.01 (27.6-28.43)[b]	28.04 (27.63-28.45)[b]	28.59 (28.16-29.02)
Diabetes mellitus, %	17.1	19.7	16.4	16.9
Fasting glucose, mg/dL	108.3 (105.9-110.7)	106.2 (103.9-108.6)	104.7 (102.6-106.9)	105.6 (5.63-5.75)
HbA1c, %	5.7 (5.64-5.75)	5.74 (5.68-5.81)	5.69 (5.63-5.75)	5.69 (5.63-5.75)
Smoker, %	38.6	40.9	46.4	49.3
Cotinine, serum, ng/mL	0.18 (0.14-0.23)	0.19 (0.15-0.25)	0.29 (0.22-0.38)	0.43 (0.33-0.57)[c,e,f]
Hypertension, %	39.7	42	43.3	44.4
Systolic blood pressure, mmHg	121.9 (120.9-123.1)	122.9 (121.8-124.1)	123 (121.8-124.1)	122.3 (121.1-123.5)
Diastolic blood pressure, mmHg	68.73 (67.93-69.53)	68.53 (67.76-69.31)	68.79 (67.94-69.64)	68.95 (68.1-69.82)
Dyslipidemia, %	37.6	41	43.4	40.2
Total cholesterol, mg/dL	185.3 (182.2-187.9)	186.9 (184.1-189.8)	186.7 (184.1-189.4)	185-9 (183.3-188.6)
BPS, μg/g creatinine	0.48 (0.44-0.51)	0.5 (0.46-0.54)	0.55 (0.51-0.59)	0.54 (0.50-0.58)

[a]$P < 0.05$, significant differences with respect to group Q1.
[b]$P < 0.01$, significant differences with respect to group Q1.
[c]$P < 0.001$, significant differences with respect to group Q1.
[d]$P < 0.0001$, significant differences with respect to group Q1.
[e]$P < 0.05$, significant differences between Q2 and Q4.
[f]$P < 0.05$, significant differences between Q3 and Q4.
The results are expressed as percentages (%) or as geometric mean (95%CI). BMI: Body mass index; HbA1c: Hemoglobin A1c; BPS: Bisphenol S; BPF: Bisphenol F.

Table 4 Simple linear regression with hemoglobin A1c and fasting glucose

	HbA1c			Fasting glucose		
Variable	Adjusted R^2	β_0	β	Adjusted R^2	β_0	β
[1]BPS	0.005[d]	5.835[d]	0.069[d]	0.006[c]	111.69[d]	2.48[d]
[1]BPF	0.000	5.795	0.006	0.000	109.65	-0.39

[c]$P < 0.001$.
[d]$P < 0.0001$.
[1]Log transformed.
HbA1c: Hemoglobin A1c; BPS: Bisphenol S; BPF: Bisphenol F.

observed that the relationship was more significant in the case of female fetuses.

The present study determined that there was a significant relationship between urinary BPS and diabetes. However, such a relationship was not observed with urinary BPF. From a molecular point of view, it is interesting to note that BPF, like BPA, has carbon and hydrogen atoms, while BPS also contains sulfur atoms[19]. There is conflicting evidence in the academic literature between BPA and diabetes. Thus, some studies observed a positive and significant relationship with diabetes mellitus[35, 36] or prediabetes[37], while others did not find a significant relationship[38]. In addition, there are even works, such as that by Wang *et al*[39], in which they determined that pregnant women with higher levels of urinary BPA had a lower risk of developing gestational diabetes.

Interestingly, both BPS and BPF (like BPA) have been shown to have pro-estrogenic and anti-androgenic activity[40]. In pancreatic cell cultures, it has been observed that both BPS and BPF can negatively affect insulin secretion and ion channels through a signaling mechanism that includes estrogen receptor beta[16]. A recent animal study conducted by Qiu *et al*[41] observed that BPF and BPS produced similar effects on the immune system in zebrafish. In an experimental non-obese diabetic

Table 5 Association between phenols and diabetes

	Diabetes	
Variable	OR (95%CI)	*P* value
[1]BPS (1)	1.115 (1.038-1.196)	0.003
[1]BPS (2)	1.109 (1.026-1.198)	0.009
[1]BPS (3)	1.099 (1.016-1.188)	0.018
[1]BPF (1)	1.020 (0.961-1.083)	0.513
[1]BPF (2)	1.005 (0.941-1.072)	0.890
[1]BPF (3)	0.991 (0.928-1.059)	0.795

[1]Log-transformed.
(1) Individual; (2) Corrected for age, sex, and body mass index; and (3) Corrected for the above parameters and smoking, hypertension, and dyslipidemia.
OR: Odds ratio; BPS: Bisphenol S; BPF: Bisphenol F.

Table 6 Association between diabetes and bisphenol S or F quartile

	Q1	Q2	Q3	Q4
Variable	Ref.	OR (95% CI)	OR (95%CI)	OR (95%CI)
[1]Diabetes (1)	Ref.	1.1 (0.86-1.41)	1.23 (0.96-1.57)	1.39 (1.09-1.77)[c]
[1]Diabetes (2)	Ref.	1.11 (0.85-1.46)	1.14 (0.87-1.48)	1.32 (1.01-1.71)[a]
[1]Diabetes (3)	Ref.	1.09 (0.83-1.43)	1.12 (0.86-1.47)	1.28 (0.99-1.67)
[2]Diabetes (1)	Ref.	1.19 (0.94-1.51)	0.95 (0.74-1.21)	0.98 (0.77-1.26)
[2]Diabetes (2)	Ref.	1.16 (0.90-1.51)	0.88 (0.68-1.15)	0.94 (0.72-1.23)
[2]Diabetes (3)	Ref.	1.17 (0.90-1.52)	0.86 (0.66-1.13)	0.92 (0.70-1.20)

[a]$P < 0.05$.
[c]$P < 0.001$.
[1]Bisphenol S (group 2).
[2]Bisphenol F (group 3).
(1) Individual; (2) Corrected for age, sex, and body mass index; and (3) Corrected for the above parameters and smoking, hypertension, and dyslipidemia.
OR: Odds ratio.

mouse model, it has been observed that BPS could negatively affect glucose homeostasis in males, while a protective effect was observed in females[17]. From a mechanistic point of view, bisphenols have the potential to affect the development of diabetes through different pathways. In addition to the classical estrogen receptors (ER-α, ER-β, and G protein-coupled receptor 30), BPA has been shown to have increased binding capacity to the estrogen-related receptor (ERR-γ)[43]. ERR-γ is important in diabetes since it plays an essential role in correctly maturing pancreatic β cells[44] and insulin secretion[45]. This receptor also plays a vital role in coordinating metabolic and endocrine signals, regulating hepatic glucose metabolism[46]. Previous work by our group demonstrated that this receptor participates in the loss of podocyte adhesion induced by BPA and is directly related to diabetic nephropathy[3]. On the other hand, it has been observed that both BPA and BPS can affect insulin cell signaling in skeletal muscle and adipose tissue (reducing the expression of insulin receptor substrate 1 and Akt phosphorylation)[43].

The linear regression model of the present work showed very significant values only with the BPS. However, the *R*-squared value was low (0.005) despite being significant. This data implies that the relationship between both variables is low; urinary BPS could only explain a tiny part of diabetes cases. Subsequent binomial and multinomial logistic regression models confirmed and reinforced the relationship between BPS and diabetes while ruling out the statistical relationship with urinary BPF. Nowadays, the vision of "one factor-one disease" could be considered obsolete. Numerous pathologies, such as diabetes, cannot be explained by the action of a single element since they are multifactorial. Therefore, the main idea extracted from the results is that BPS is an environmental factor related to diabetes.

Table 7 Association between hypertension or dyslipidemia and bisphenol S or F

	Hypertension		Dyslipidemia	
Variable	OR (95%CI)	*P* value	OR (95%CI)	*P* value
[1]BPS (1)	1.12 (1.06-1.18)	0.000	0.99 (0.99-1.11)	0.099
[1]BPS (2)	1.09 (1.02-1.17)	0.007	1.02 (0.95-1.08)	0.607
[1]BPS (3)	1.08 (1.01-1.16)	0.017	0.99 (0.937-1.065)	0.980
[1]BPF (1)	1.07 (1.02-1.12)	0.005	1.05 (1.005-1.1)	0.03
[1]BPF (2)	1.06 (1.001-1.12)	0.044	1.04 (0.98-1.1)	0.168
[1]BPF (3)	1.04 (0.99-1.11)	0.136	1.03 (0.98-1.09)	0.274

[1]Log-transformed.
(1) Individual; (2) Corrected for age, sex, and body mass index; and (3) Corrected for the above parameters and smoking, hypertension, and dyslipidemia.
OR: Odds ratio.

On the other hand, complementary studies on hypertension and dyslipidemia have shown interesting evidence. First, both derived compounds show interesting significant relationships with the risk of hypertension, especially BPS. As with diabetes, few works study the relationship between BPS or BPF and these diseases. Jiang *et al*[42] found a positive and significant relationship between individuals with higher levels of urinary BPS, but not BPF, with hypertension. On the other hand, the works of Liu *et al*[22] and Jacobson *et al*[24] found a significant relationship between urinary BPF[22] or both phenolic derivatives[24] and obesity in children and adolescents.

Due to the differences observed in the risks of predisposition to diseases, it could be stated that the compounds derived from BPA (despite having similar hormonal activity) could act on different cell signaling mechanisms, promoting the development or progression of different diseases.

CONCLUSION

The present study has determined a strong relationship between urinary BPS, not BPF, and diabetes risk. In the case of hypertension, both molecules could be involved in pathophysiological mechanisms, which, in the case of dyslipidemia, would be exclusive to BPF. Future studies will be necessary to delve into the paradigm and explore the relationship of the new BPA-derived molecules with other related diseases, such as kidney disease. BPA substitute molecules do not exempt the population from potential health risks.

ARTICLE HIGHLIGHTS

Research background

New restrictions on the use of bisphenol A (BPA) have conditioned the use of new derivative compounds by the plastics industry. The small amount of evidence for its possible effects on human health shows its need, especially in diseases such as diabetes, whose incidence has increased substantially in recent years.

Research motivation

The study of the urinary excretion of the new bisphenols and their possible relationship with human health is of particular importance. The present work aimed to provide new evidence that supports the need for restriction in using new molecules derived from BPA.

Research objectives

The work's objective was to analyze the relationship between urinary bisphenols and diabetes in one of the largest global cohorts, National Health and Nutrition Examination Survey (NHANES). The possible results could support the need to explore the signaling pathways involved in the pancreatic pathophysiology potentially induced by this class of molecules.

Research methods

By applying descriptive statistics, simple linear regressions, and logistic regression models, this study

aimed to analyze the data from the NHANES cohort in a novel way in a context that has been little studied in the academic literature.

Research results

After using all the tools and statistical models, the results have consistently pointed to bisphenol S as a risk factor for diabetes, excluding bisphenol F. On the other hand, the relationships observed with hypertension and dyslipidemia maintain the need to evaluate both molecules in the human health context.

Research conclusions

In a novel way in the NHANES cohort, the present study has shown that exposure to new bisphenols is directly related to diabetes.

Research perspectives

Future research should explore the causal relationship through longitudinal studies and evaluate the potential deleterious effects on other pathologies, such as kidney disease.

FOOTNOTES

Author contributions: Moreno-Gómez-Toledano R contributed to the conceptualization and writing of original draft; Moreno-Gómez-Toledano R, Vélez-Vélez E, Arenas MI, Saura M, and Bosch RJ contributed to the data curation, and manuscript writing, review, and editing; Moreno-Gómez-Toledano R and Vélez-Vélez E contributed to the formal analysis and methodology; Saura M and Bosch RJ contributed to the funding acquisition and project administration; Moreno-Gómez-Toledano R, Vélez-Vélez E, Arenas MI, Saura M, and Bosch RJ contributed to the investigation; all authors have read and agreed to the published version of the manuscript.

Supported by the Instituto de Salud Carlos III, No. PI15/02139; and Comunidad Autónoma de Madrid, No. B2017-BMD-3686.

Institutional review board statement: The study was reviewed and approved for publication by our Institutional Reviewer.

Conflict-of-interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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S-Editor: Zhang H
L-Editor: Wang TQ
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 532-542

DOI: 10.4239/wjd.v13.i7.532

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Efficacy and mechanism of anti-vascular endothelial growth factor drugs for diabetic macular edema patients

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Specialty type: Ophthalmology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Hilliard ME, United States; Kilanowska A, Poland; Ortega AL, Spain

Received: February 17, 2022
Peer-review started: February 17, 2022
First decision: April 17, 2022
Revised: April 26, 2022
Accepted: June 16, 2022
Article in press: June 16, 2022
Published online: July 15, 2022

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Abstract

BACKGROUND

Diabetes is a serious public health concern in China, with 30% of patients developing retinopathy, and diabetic macular edema (DME) having the biggest impact on vision. High blood glucose level can cause retinal cell hypoxia, thus promoting vascular endothelial growth factor (VEGF) formation and increasing vascular permeability, which induces DME. Moreover, cell hypoxia can accelerate the rate of apoptosis, which leads to the aging of patients. In severe cases, optic cell apoptosis or retinal fibrosis and permanent blindness may occur.

AIM

To investigate and compare the efficacy, mechanism, and differences between two anti-VEGF drugs (Compaq and ranibizumab) in DME patients.

METHODS

Ninety-six patients with DME who attended our hospital from April 2018 to February 2020 were included and randomly divided into two groups (Compaq group and ranibizumab group). The groups received vitreal cavity injections of 0.5 mg Compaq and 0.5 mg ranibizumab, respectively, once a month. The best corrected visual acuity (BCVA), intraocular pressure (IOP), macular retinal thickness (CMT), macular choroidal thickness (SFCT), foveal no perfusion area (FAZ), superficial capillary density, deep capillary density, treatment effect, and adverse reactions were compared before and after treatment and between the two groups.

RESULTS

Before treatment and 1-mo post-treatment, there was no statistically significant

difference in the estimated BCVA in both groups ($P > 0.05$). BCVA decreased in the Compaq group 3 mo after treatment, and the difference was statistically significant ($P < 0.05$). Before treatment, and 1 mo and 3 mo post-treatment, there was no statistically significant difference in the estimated IOP in either group ($P > 0.05$). Before treatment and 1-mo post-treatment, there was no statistically significant difference in the estimated CMT, SFCT, or FAZ in either group ($P > 0.05$). CMT and SFCT values decreased in the Compaq group 3 mo post-treatment, and the difference was statistically significant ($P < 0.05$). Before treatment, and 1 mo and 3 mo post-treatment, there were no statistically significant differences in vascular density in the shallow or deep capillary plexi of the fovea, parafovea, or overall macular area between the two groups ($P > 0.05$). Marked efficient, effective, and invalid rates were 70.83% and 52.08%, 27.08% and 39.58%, and 2.08% and 8.33% in the Compaq and ranibizumab groups, respectively. The differences between the two groups were statistically significant ($P < 0.05$).

CONCLUSION

Anti-VEGF drugs can effectively improve CMT and SFCT, without affecting microcirculation, thus providing an effective and safe treatment for patients with DME.

Key Words: Diabetic macular edema; Vascular endothelial growth factor; Compaq; Ranibizumab; Optimally correct vision; Diabetes

Core Tip: The main pathological feature of diabetic macular edema (DME) is abnormal neovascularization throughout the retinal pigment epithelium. New vessels develop rapidly and are fragile, thus resulting to rupture and retinal detachment, macular edema, impaired, vision and blind spots. Without effective treatment, vision declines rapidly, causing irreversible impairment. Compaq has a strong affinity with vascular endothelial growth factor (VEGF) receptors, and as a novel VEGF biological agent, it has a relatively strong inhibition of vascular growth in ocular lesions. Our study investigated the effect and mechanism of anti-VEGF drugs in DME patients to improve clinical DME treatment.

Citation: Li YF, Ren Q, Sun CH, Li L, Lian HD, Sun RX, Su X, Yu H. Efficacy and mechanism of anti-vascular endothelial growth factor drugs for diabetic macular edema patients. *World J Diabetes* 2022; 13(7): 532-542
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/532.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.532

INTRODUCTION

Diabetic macular edema (DME), which manifests clinically as visual impairment, is a common complication of diabetes[1-3]. Long-term high blood glucose is the basis of DME, as it causes increased endothelial cell permeability in the tight junctions of retinal capillaries, allowing protein and fluid to accumulate in the area of the macula, resulting in macular edema. Previous studies have also found that along with leaking capillary endothelial cells, an increase in glycosylated hemoglobin due to high blood sugar is also a risk factor for cystoid macular edema. Strict blood glucose control within a reasonable range can slow down the development of DME.

Vascular endothelial growth factor (VEGF) play an important role in the pathophysiology of DME[4]. In a high blood glucose environment, glycosylation products increase, and active oxygen is at a relatively high level, leading to further diglyceride production. This activates protein kinase C, which mediates the generation of VEGF[5]. VEGF is the primary regulatory factor for neovascularization and vascular permeability, which characterizes DME. The interaction of vascular receptors on the endothelial cell surface can be inactivated through VEGF inhibition to prevent vascular endothelial hyperplasia, thereby reducing retinal neovascularization and blood vessel leakage in the macular area. Currently, according to evidence from multiple regions, the pharmaceutical drug Compaq (Chengdu Kanghong Biotechnology Co., LTD., Chengdu, China; National Drug approval S20130012)[6] acts directly on new vessels in retinal lesion tissue, thus reducing the incidence of blood vessel hyperplasia caused by photocoagulation. Moreover, inreverse rate of retinal tissue can be reduced by laser photocoagulation reduction. For these reasons, anti-VEGF drugs are recommended as the first-line treatment for DME to improve clinical manifestations. Examples of anti-VEGF drugs used for macular edema in China include bevacizumab, Compaq, and ranibizumab[7]. Vitreal cavity injection is the preferred route of administration for anti-VEGF drugs.

Compaq is a novel anti-VEGF drug developed independently in China, which can specifically bind to VEGF-A, VEGF-B, and placenta growth factors to inhibit the activation of VEGF families, thus preventing neovascularization and reducing blood retinal barrier (BRB) damage and minor microvasculature leakage. Studies have shown[8,9] Compaq as an efficacious DME treatment, but whether it can inhibit neovascularization, reduce the perfusion area in the macular area, and improve microcirculation remains unclear.

There are a few studies comparing the effects of Compaq with that of ranibizumab for DME administered *via* vitreal cavity injection. For this study, we used optical coherence tomography angiography (OCTA) to measure the treatment effect and mechanism of action in these two anti-VEGF drugs. Based on the segment frequency amplitude of B-scan correlation calculation and the real-time flow of red blood cells in retinal vascular correlation calculation, OCTA can remove the correlation of fixed organization, highlight regular blood flow images of the organization, and recombine all images to obtain structures, such as the retina and choroid blood vessels, to facilitate blood flow evaluation[10].

MATERIALS AND METHODS

Patients

A total of 96 patients with DME who visited our hospital from April 2018 to February 2020 were included in the study. Patients were randomly divided into two groups: Compaq (n = 48) and ranibizumab (n = 48) by assigning even and odd-numbered sequences. The inclusion criteria were as follows: (1) Age 54–79 years; (2) Diagnosed according to the criteria and treatment guidelines for diabetic retinopathy (DR)[11] (with DME within the proliferative phase of the DR lesion stage); (3) Hospital admission and receipt of fundoscopic angiography, with diagnosis by OCTA examination; (4) Retinal fovea thickness in the macular area > 250 μm; and (5) Ocular IOP range: 10-21 mmHg. The exclusion criteria were as follows: (1) Presence of eye tumors; (2) History of ocular trauma; (3) Retinal macular spots and tissue hyperplasia; (4) Reticular vein obstruction and senile macular lesions; (5) Hypertensive retinopathy; (6) Combined cataract and glaucoma; and (7) Other systematic major disease.

The study was approved by the Medical Ethics Committee of our hospital, and informed consent from the patients' families was obtained for the treatment plan of this study.

Therapeutic regimen

Patients were placed in the supine position, and the cornea and conjunctival sac were cleaned. Routine pre-anesthesia operations were performed, followed by surface anesthesia. Under the microscope (Nikon, Tokyo, Japan), the doctor opened the patient's eyelid. Compaq (0.10 mg/mL, 0.2 mL/injection) was injected into the vitreous chamber of patients in both groups. After injection, the needle was withdrawn slowly, and 0.5 mg/eye/time was infused in the vitreous cavity once per month for the first 3 mo (0.05 mL), followed by intravitreal administration once every 3 mo.

Basic treatment

All patients underwent lacrimal tract irrigation 3 d before surgery and were treated with levofloxacin eye drops four times a day for 5 d (Shentian Pharmaceutical Co., LTD. Noto Factor, Japan; National medicine approval number J20150106; 10 mL/branch/box) after surgery.

Observation indexes and evaluations

The best corrected visual acuity (BCVA), intraocular pressure (IOP), macular retinal thickness (CMT), macular choroidal thickness (SFCT), foveal no perfusion area (FAZ), superficial capillary density, deep capillary density, treatment effect, and adverse reactions were compared between the two groups before and after treatment.

BCVA was measured using a TDRS visual acuity chart at a distance of 4 m, and the maximum number of letters obtained was recorded at four timepoints: pre-treatment, and at 1, 3, and 6 mo post-treatment.

For IOP measurement, a non-contact ophthalmometer (model AD-1900; Neusoft Xikang Co., LTD., Shenyang, China) was used, and the average value of three inspections were taken.

Astigmatism was examined using the slit slice method, and an OCT instrument (American BD company production, model AU-300; GE Company, Chicago, Illinois) was used to scan the macular area within a range of 6 mm × 6 mm. Consequently, the retinal thickness of the macular fovea was measured.

Treatments were evaluated with reference to the diagnosis and treatment of diabetic retinopathy[12]. Treatments were considered markedly effective if edema had disappeared, retinal hemorrhage had been completely absorbed, and there was no obvious neovascularization, leakage, or perfusion in the macular area 3 mo after treatment. Treatments were considered effective if retinal hemorrhage had partially absorbed, vascular leakage was reduced, the perfusion density (PD) in the non-perfusion area was < 5, and no new vessels were assumed to be functioning. Lastly, treatments were considered invalid if patients did not meet the above criteria.

Ophthalmic testing included the following: (1) Routine examination: BCVA was measured using an international standard visual acuity chart; IOP was measured using a non-contact tonometer; and (2) OCT examination: A Zeiss Cirrus 5000 OCT instrument (Carl Zeiss Meditec, Jena, Germany) was used. CMT was measured with 2 PD on the optic disc temporal side and 1.5 PD below the macular fovea. Three measurements were obtained, and the average value was considered. SFCT was measured using the caliper function of the instrument. The retinal blood flow imaging mode was selected, and the scanning range of the macular area was 3 mm × 3 mm, scanning signal intensity index was > 45, and transverse and longitudinal scanning required 3 s. The patients were instructed not to move their eyes during the scan. Supporting analysis software was used to measure the vessel density of the shallow capillary plexus (SCP) and deep capillary plexus (DCP) in the FAZ within the scanning range of 3 mm × 3 mm in the macular area at the SCP level. All examinations were performed by the same physician and reviewed independently by two surgeons clinically experienced fundus imaging analysis.

Statistical analysis

A normal distribution test showed that the BCVA values of the patients in this study were consistent with an approximate normal distribution or normal distribution, and was expressed as mean ± SD. Test was used for comparisons between groups. The counting data were expressed as percentages, and comparisons were based on χ^2 test or the Mann–Whitney U test. Professional SPSS 21.0 software (IBM Corp., Armonk, NY) was used for data processing, and the significance level was set at $\alpha = 0.05$.

RESULTS

Baseline data between the two groups

The age, sex, body mass index, and BCVA before treatment and the distribution of the affected side were compared between the two groups, and no statistically significant difference was found ($P > 0.05$), as shown in Table 1.

Comparison of estimated BCVA and IOP values between the two groups

Before and 1-mo post-treatment, there was no statistically significant difference between the estimated value of BCVA in either group ($P > 0.05$). After 3 mo, a decrease was observed in the Compaq group, and the difference was statistically significant ($P < 0.05$). However, there was no statistically significant difference in the estimated value of IOP before, 1 mo, or 3 mo after the treatment in either group ($P > 0.05$), as shown in Table 2.

Comparison of estimated CMT, SFCT, and FAZ values between the two groups

There were no statistically significant differences in the estimated values of CMT, SFCT, or FAZ before or 1-mo post-treatment in either group ($P > 0.05$). Three months post-treatment, the estimated values of CMT and SFCT in the Compaq group were lower than those in the ranibizumab group, and the difference was statistically significant ($P < 0.05$) (Table 3).

Comparison of vascular density in the SCP and DCP between the two groups

Before, 1 mo, and 3 mo post-treatment, there were no statistically significant differences in the vascular density of the SCP and DCP of the fovea, parafovea, or overall macular area between the Compaq and ranibizumab groups ($P > 0.05$), as shown in Table 4 and Table 5.

Comparison of clinical efficiency between the two groups

Three months post-treatment, the rates of marked efficiency, effective, and invalid in the Compaq and ranibizumab groups were 70.83% and 52.08%, 27.08% and 39.58%, and 2.08% and 8.33%, respectively, and the difference between the two groups was statistically significant ($P < 0.05$), as shown in Table 6 and Figure 1A.

Comparison of adverse reaction rates between the two groups

The rates of adverse reactions in the Compaq and ranibizumab groups were 6.25% and 12.50%, respectively, and there was no statistically significant difference between the two groups ($P > 0.05$), as shown in Table 7 and Figure 1B.

Typical cases

A 71-year-old male patient, with a history of diabetes over 12 years, reported a significant decrease in visual acuity in the prior 6 mo. After admission, he was diagnosed with diabetic macular edema by fundus angiography and optical coherence tomography. Before treatment, the foveal thickness in the macular area was > 477.2 μm, and the BCVA value was 0.83 LogMAR. The patient was treated with intravitreal injection of Conbercept 0.5 mg once a month. The OCT examination results before and after treatment are shown in Figure 2. The patient's visual acuity BCVA recovered to 0.55 LogMAR 3 mo after

Table 1 Baseline data between the two groups

Baseline data	Compaq group (*n* = 48)	Ranibizumab group (*n* = 48)	t/χ^2 value	*P* value
Age (yr)	64.8 ± 7.2	66.3 ± 6.9	-1.042	0.300
BMI (kg/m^2)	23.5 ± 2.3	23.2 ± 2.8	0.574	0.568
Before treatment: BCVA (LogMAR)	0.78 ± 0.12	0.80 ± 0.11	-0.851	0.397
Gender, *n* (%)			2.043	0.153
Male	27 (56.25)	20 (41.67)		
Female	21 (43.75)	28 (58.33)		
Distribution of affected side, *n* (%)			0.667	0.414
Left	22 (45.83)	26 (54.17)		
Right	26 (54.17)	22 (45.83)		

BCVA: Best corrected visual acuity.

Table 2 Comparison of estimated values of best corrected visual acuity, intraocular pressure between the two groups (mean ± SD)

Groups	BCVA (LogMAR)			IOP (mmHg)		
	Before treatment	1 mo after treatment	3 mo after treatment	Before treatment	1 mo after treatment	3 mo after treatment
Compaq group (*n* = 48)	0.78 ± 0.12	0.72 ± 0.13	0.51 ± 0.10	16.84 ± 2.77	16.40 ± 2.81	16.39 ± 2.64
Ranibizumab group (*n* = 48)	0.80 ± 0.11	0.75 ± 0.14	0.57 ± 0.13	16.50 ± 2.80	16.72 ± 2.76	16.81 ± 2.82
t value	-0.851	-1.088	-2.535	0.598	-0.563	-0.753
P value	0.397	0.279	0.013	0.551	0.575	0.453

BCVA: Best corrected visual acuity; IOP: Intraocular pressure.

treatment.

DISCUSSION

In diabetes, the retina is prone to injury due to oxidative stress. A high blood glucose environment can increase active oxygen levels as well as oxygen production in the mitochondria, thus impeding balance between the deoxidation and neutralization of mitochondrial reactive oxygen species in the body. This increased oxidative stress level results in macular edema[13,14]. Research has verified that the excessive production of mitochondrial reactive oxygen species and the decrease of antioxidant enzymes promote the progression of diabetes.

As a novel anti-VEGF, developed independently in China, Compaq can be used to inhibit the increase of vascular wall permeability and neovascularization. The VEGF concentrations in the vitreous chamber and perivascular vessels on the retinal surface are abnormally elevated in DME patients, resulting in retinal neovascularization and increased vascular wall permeability, finally leading to edema. Our study showed that before treatment and 1-mo post-treatment, there was no statistical difference in BCVA between the two groups. However, 3 mo post-treatment, BCVA was significantly lower in the Compaq group than in the ranibizumab group.

Various kinds of anti-VEGF drugs are commonly used in DME treatment, including imported drugs, such as ranibizumab. These drugs bind to and inhibit VEGF receptors to prevent the formation of specific receptors of neovascularization, and therefore, blood glucose and its effects such as retinal capillary permeability can be reduced to improve vision[15]. Compaq eye injections have a significant effect on neovascularization inhibition to reduce VEGF concentration and vascular wall permeability in the eyes and reduce the infiltration of blood vessels; therefore, retinal edema can be absorbed and the degree of macular edema can be relieved to significantly improve visual performance. At present, Compaq is used globally in the field of ophthalmology to reduce macular central retina thickness and choroid thickness of the macular fovea to improve vision in DME patients. Compaq treatment has been

Table 3 Comparison of estimated values of macular retinal thickness, macular choroidal thickness, foveal no perfusion area between the two groups (mean ± SD)

Groups	Before treatment	1 mo after treatment	3 mo after treatment
CMT (μm)			
Compaq group (*n* = 48)	445.8 ± 89.6	372.1 ± 76.0	210.6 ± 66.4
Ranibizumab group (*n* = 48)	452.7 ± 93.2	384.0 ± 80.6	243.1 ± 73.5
t value	-0.370	-0.744	-2.273
P value	0.712	0.459	0.025
SFCT (μm)			
Compaq group (*n* = 48)	335.1 ± 55.9	323.4 ± 59.5	281.6 ± 54.0
Ranibizumab group (*n* = 48)	340.5 ± 58.3	330.5 ± 63.0	306.2 ± 57.3
t value	-0.463	-0.568	-2.165
P value	0.644	0.572	0.033
FAZ (mm^2)			
Compaq group (*n* = 48)	0.74 ± 0.10	0.72 ± 0.12	0.73 ± 0.11
Ranibizumab group (*n* = 48)	0.75 ± 0.12	0.74 ± 0.14	0.74 ± 0.11
t value	-0.444	-0.751	-0.445
P value	0.658	0.454	0.657

CMT: Macular retinal thickness; SFCT: Macular choroidal thickness; FAZ: Foveal no perfusion area.

Table 4 Comparison of vascular density in the shallow capillary plexus between the two groups (mean ± SD, %)

Groups	Before treatment	1 mo after treatment	3 mo after treatment
Fovea			
Compaq group (*n* = 48)	20.64 ± 4.40	20.30 ± 3.95	20.28 ± 3.77
Ranibizumab group (*n* = 48)	20.90 ± 4.83	20.48 ± 4.20	20.37 ± 4.14
t value	-0.276	-0.216	-0.111
P value	0.783	0.829	0.912
Parafovea			
Compaq group (*n* = 48)	38.56 ± 4.82	38.10 ± 4.50	37.73 ± 4.72
Ranibizumab group (*n* = 48)	39.10 ± 5.57	38.67 ± 5.53	38.38 ± 5.28
t value	-0.508	-0.554	-0.636
P value	0.613	0.581	0.526
Overall macular area			
Compaq group (*n* = 48)	35.74 ± 5.10	35.43 ± 4.85	34.92 ± 5.51
Ranibizumab group (*n* = 48)	36.30 ± 5.34	35.67 ± 5.11	34.58 ± 5.18
t value	-0.525	-0.236	0.311
P value	0.601	0.814	0.756

proven to be effective and safe[16].

DME treatment of the retina and choroid can sometimes lead to retinal capillary cell loss and degeneration and vascular endothelial cell hyperplasia in diabetes patients, thereby destroying the blood-retinal barrier[17]. Meanwhile, VEGF secretion can be increased, leading to retinal tight junction dysfunction and pericyte loss, as well as an impaired BRB; thus, vascular wall permeability can be increased, causing fluid to leak into retinal tissue and accumulate, resulting in macular edema. Severe cases may develop macular edema, thickening, vision loss, or even blindness. Some studies have found

Table 5 Comparison of vascular density in the deep capillary plexus between the two groups (mean ± SD, %)

Groups	Before treatment	1 mo after treatment	3 mo after treatment
Fovea			
Compaq group (n = 48)	18.58 ± 3.80	18.23 ± 3.75	17.86 ± 4.12
Ranibizumab group (n = 48)	19.14 ± 4.00	18.78 ± 4.24	18.47 ± 3.96
t value	-0.703	-0.673	-0.740
P value	0.484	0.502	0.461
Parafovea			
Compaq group (n = 48)	40.92 ± 5.73	40.51 ± 4.85	40.38 ± 5.22
Ranibizumab group (n = 48)	40.40 ± 5.51	40.10 ± 5.28	39.56 ± 4.87
t value	0.453	0.396	0.796
P value	0.651	0.693	0.428
Overall macular area			
Compaq group (n = 48)	39.64 ± 4.85	39.40 ± 4.77	38.78 ± 4.62
Ranibizumab group (n = 48)	40.43 ± 5.18	39.93 ± 5.03	39.52 ± 4.85
t value	-0.771	-0.530	-0.765
P value	0.442	0.598	0.446

Table 6 Comparison of clinical efficiency between the two groups, n (%)

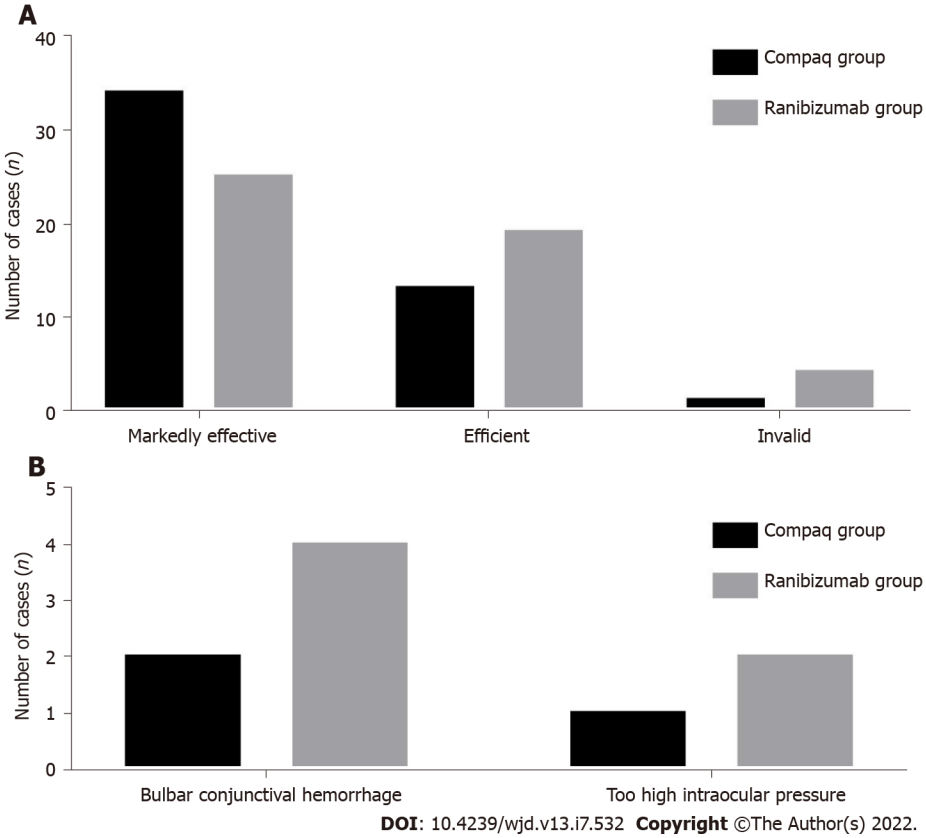
Groups	Markedly efficiency	Efficient	Invalid
Compaq group (n = 48)	34 (70.83)	13 (27.08)	1 (2.08)
Ranibizumab group (n = 48)	25 (52.08)	19 (39.58)	4 (8.33)
Z value	-1.993		
P value	0.046		

Table 7 Comparison of incidence of adverse reaction between the two groups, n (%)

Groups	Bulbar conjunctival hemorrhage	Too high intraocular pressure	Adverse reaction
Compaq group (n = 48)	2	1	3 (6.25)
Ranibizumab group (n = 48)	4	2	6 (12.50)
χ^2 value			1.333
P value			0.248

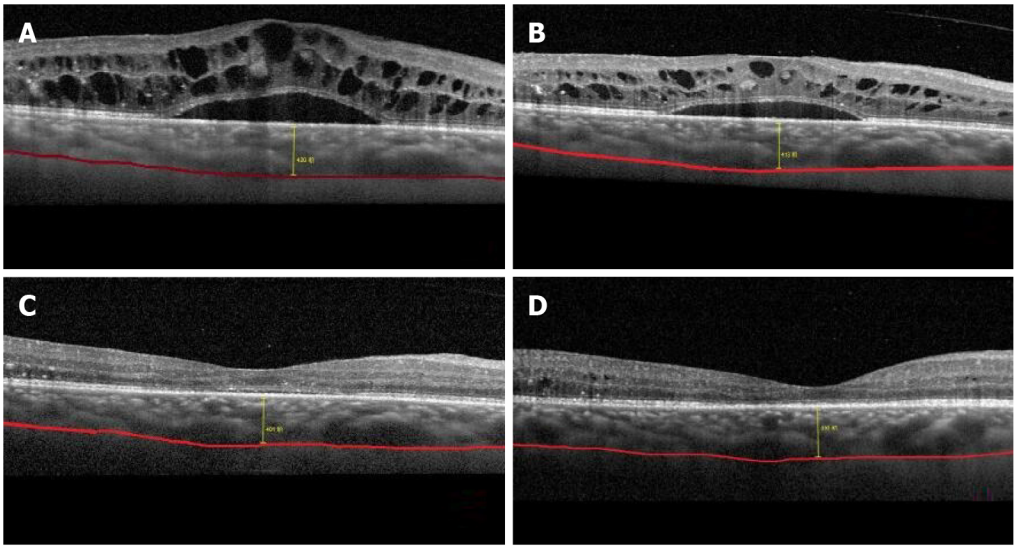
that DR patients may have a relatively thin choroid compared to DME patients. Upon OCTA examination, the thicknesses and changes in each retinal layer can be clearly observed, and the structural image and thickness of the choroid can be distinguished[18]. However, according to our study results, before treatment and 1-mo post-treatment, there was no statistical difference in CMT, SFCT, or FAZ level between the two groups. Three months post-treatment, the estimated values of CMT and SFCT in the Compaq group were significantly lower than those in the ranibizumab group.

Moreover, previous studies have shown that increased total cholesterol, triglycerides, and low-density lipoprotein is related to DME in diabetic patients. Anti-VEGF administered through vitreal cavity injection can improve glucose and lipid levels and decrease levels of oxidative stress throughout the body, which significantly reduces VEGF production, thus reducing retinal vein and artery diameter. This also reduces the permeability of retinal capillaries, inhibiting neovascularization and reducing the extent of damage in the BRB. The thicknesses of the macular central retina and choroid can be reduced once macular edema is relieved. After anti-VEGF treatment in DME, the detailed reasons for decreased macular central choroid thickness remain unclear, which may be because anti-VEGF drugs such as Compaq can inhibit signaling, and the activity of VEGF can be inhibited by binding to VEGF to reduce



DOI: 10.4239/wjd.v13.i7.532

Figure 1 Histogram of clinical efficiency and incidence of adverse reaction between the two groups. A: Histogram of clinical efficiency; B: Incidence of adverse reaction.



DOI: 10.4239/wjd.v13.i7.532

Figure 2 The optical coherence tomography test results of before and after patient treatment. A: The test result of optical coherence tomography before the treatment, where macular edema was obvious; B: Re-examination after 1 wk of treatment, where the macular edema is slightly relieved; C: Re-examination after 1 month of treatment, where the macular edema was significantly reduced; D: The condition of the patient 3 mo post-treatment, where the macular edema has nearly disappeared, and the choroid thickness has become significantly thinner.

respiration and edema caused by retinal vascular leakage, resulting in decreased macular central retina and choroid thickness for improved vision.

DME is a complex pathologic progress caused by multiple factors. Currently, it is believed that microvascular lesion in diabetes can cause blood flow changes in retinal microvasculature, and histanoxia can lead to aggravated inflammation, resulting in the release of various inflammatory factors,

such as prostaglandins, leukocyte trienes, intercellular adhesion molecule-1, integrin, and tumor necrosis factor α. These factors cause vascular endothelial injury and increased VEGF secretion, thus promoting macular edema[19]. Conversely, macular edema can aggravate histanoxia, and VEGF can be further increased to stimulate the growth of new blood vessels, as well as the formation of a microaneurysm, which becomes a negative feedback loop. Our study showed that, before and after treatment, there were no statistical differences in vascular density in the SCP and DCP in the overall macular area between the two groups. There was also no significant difference in treatment efficacy, indicating that the two drugs selected in our study had no influence on microcirculation, and were safe. Compaq can directly act on new blood vessels in retinal lesions and reduce the damage caused by laser photocoagulation on the retina, thereby resulting in a decreased inflammatory response rate in retinal tissue.

Some studies have shown that both ranibizumab and Compaq can reduce the macular FAZ area and increase vascular density in the SCP to improve microcirculation[20]. With the progression of diabetes, VEGF secretion can be abnormally increased and BRB irreversibility can be aggravated, resulting in the interrupted integrity of the macular arch, and forming of the microaneurysm. Normal vascular tissue is destroyed, and the overall vascular density of the macular area is also reduced. Reports on the quantitative observation of the FAZ area and vascular density using OCTA in the treatment of DME remain unclear.

Previous studies[21] used Conbercept for treatment and found that it can exert strong affinity and multi-target characteristics in the treatment, and can reach the target concentration in a short time. Anti-VEGF therapy decreased SFCT, which has become a relevant parameter for drug selection and follow-up evaluation. DME has a very large impact on the choroid and has a very large impact on the patient's visual acuity. Anti-VEGF drugs can inhibit the biological activity of abnormal VEGF in new blood vessels in the body. From the results of this study, it can be concluded that anti-VEGF drugs can effectively improve CMT and SFCT in patients with DME, restore good visual effects, and represent a safe and efficient treatment regimen for DME. At present, there is no accurate software for measuring choroidal thickness, and the measurement of SFCT is performed by highly qualified physicians. Inevitably, there will be some errors. Automatic analysis software may reduce these errors and reduce the number of times choroidal thickness is measured.

Previous studies on the effect of anti-VEGF drugs on DME have mostly analyzed the changes in visual acuity, central macular retinal thickness, and choroid thickness, while other studies have focused on the changes and correlation in eye axis before and after treatment[22]. This study is unique in that it observed and analyzed the effects of anti-VEGF drugs on SFCT, FAZ, and microcirculation. Concurrently, it also explored the improvements in conventional indicators, such as vision and intraocular pressure, which are of high clinical value.

This study was a clinical controlled study on the treatment of DME patients with vitreous injection of ranibizumab and Compaq. During the follow-up process, we discovered that both drugs could reduce CMT and SFCT to a certain extent and improve the visual acuity of patients. However, this study was a preliminary clinical application study with a small sample size and a short follow-up period; hence, further studies are warranted to confirm our findings.

CONCLUSION

In summary, anti-VEGF drugs can effectively improve CMT and SFCT, without affecting microcirculation, thus resulting in positive treatment outcomes.

ARTICLE HIGHLIGHTS

Research background

Diabetes is a serious public health concern in China, with 30% of patients developing retinopathy, and diabetic macular edema (DME) having the biggest impact on vision.

Research motivation

Compaq as an efficacious DME treatment, but whether it can inhibit neovascularization, reduce the perfusion area in the macular area, and improve microcirculation remains unclear.

Research objectives

This study aimed to investigate and compare the efficacy, mechanism, and differences between two anti-vascular endothelial growth factor (VEGF) drugs (Compaq and ranibizumab) in DME patients.

Research methods

Total 96 patients with DME were divided into two groups with different treatment modalities.

Research results

Marked efficient, effective, and invalid rates were 70.83% and 52.08%, 27.08% and 39.58%, and 2.08% and 8.33% in the Compaq and ranibizumab groups, respectively.

Research conclusions

Anti-VEGF drugs can effectively improve macular retinal thickness and macular choroidal thickness, without affecting microcirculation.

Research perspectives

This study was a preliminary clinical application study with a small sample size and a short follow-up period; hence, further studies are warranted to confirm our findings.

FOOTNOTES

Author contributions: Li YF and Yu H designed this study; Li YF wrote this manuscript; Li YF, Ren Q, Sun ZH, Li L, Lian HD, Sun RX, Sun X and Yu H were responsible for sorting the data; and all authors read and confirmed the revision of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Shijiazhuang City People's Hospital Institutional Review Board (Approval No. 2017-22).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors have nothing to disclose.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 543-552

DOI: 10.4239/wjd.v13.i7.543

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Observational Study

Elevated levels of fructosamine are independently associated with SARS-CoV-2 reinfection: A 12-mo follow-up study

Xiao-Yan Huang, Li-Juan Yang, Xiang Hu, Xing-Xing Zhang, Xiao Gu, Lin-Jia Du, Zhi-Ying He, Xue-Jiang Gu

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ford J, United States; Gluvic Z, Serbia; Guven M, Turkey
A-Editor: Liu X, China

Received: December 8, 2021
Peer-review started: December 8, 2021
First decision: April 18, 2022
Revised: April 29, 2022
Accepted: June 13, 2022
Article in press: June 13, 2022
Published online: July 15, 2022

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Abstract

BACKGROUND

The association between blood levels of fructosamine (FMN) and recurrent coronavirus disease 2019 (COVID-19) is currently unclear.

AIM

To investigate a prospective relationship between blood levels of FMN and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) reinfection.

METHODS

A total of 146 Chinese hospitalized patients infected with SARS-CoV-2 were consecutively collectively recruited and followed from January 2020 to May 2021. Diagnosis of COVID-19 and SARS-CoV-2 reinfection was based on the diagnostic criteria and treatment protocol in China. The levels of FMN were determined in blood and divided into tertiles based on their distribution in the cohort of COVID-19 patients. Multivariate-adjusted hazard ratios (HRs) with 95% confidence intervals (CIs) were estimated for SARS-CoV-2 reinfection across the tertiles of FMN levels. A Cox regression model was used to generate the HR for SARS-CoV-2 reinfection in the participants in the top tertile of FMN levels compared with those at the bottom. Disease-free survival was used as the time variable, and relapse was used as the state variable, adjusted for age, gender, influencing factors such as diabetes mellitus, hypertension, and corticosteroid therapy, and clinical indexes such as acute liver failure, acute kidney failure, white blood cell (WBC) count, C-reactive protein, prognostic nutritional index (PNI), and blood lipids. Kaplan-Meier analysis with log-rank tests was used to compare the survival rate

between patients with elevated FMN levels (FMN > 1.93 mmol/L, the top tertile) and those with nonelevated levels.

RESULTS

Clinical data for the 146 patients with confirmed COVID-19 [age 49 (39-55) years; 49% males] were analyzed. Eleven patients had SARS-CoV-2 reinfection. The SARS-CoV-2 reinfection rate in patients with elevated FMN levels was significantly higher than that in patients with nonelevated FMN (17% *vs* 3%; $P = 0.008$) at the end of the 12-mo follow-up. After adjustments for gender, age, diabetes mellitus, hypertension, corticosteroid therapy, WBC count, PNI, indexes of liver and renal function, and blood lipids, patients with nonelevated FMN levels had a lower risk of SARS-CoV-2 reinfection than those with elevated FMN levels (HR = 6.249, 95%CI: 1.377-28.351; $P = 0.018$). Kaplan-Meier analysis showed that the cumulative survival rate of patients infected with SARS-CoV-2 was higher in patients with nonelevated FMN levels than in those with elevated FMN levels (97% *vs* 83%; log rank $P = 0.002$).

CONCLUSION

Elevated levels of FMN are independently associated with SARS-CoV-2 reinfection, which highlights that patients with elevated FMN should be cautiously monitored after hospital discharge.

Key Words: Fructosamine; COVID-19; Reinfection; Blood

Core Tip: Diabetes is a risk factor for coronavirus disease 2019 (COVID-19), which results in increased severity and mortality but has no relationship with reinfection. The present study, for the first time, reported the relationship between severe acute respiratory syndrome coronavirus 2 reinfection and blood levels of fructosamine (FMN), an index reflecting recent glycemic control. Our results demonstrated that FMN levels may influence the prognosis of patients with COVID-19, and patients with high FMN levels should be followed closely to monitor reinfection.

Citation: Huang XY, Yang LJ, Hu X, Zhang XX, Gu X, Du LJ, He ZY, Gu XJ. Elevated levels of fructosamine are independently associated with SARS-CoV-2 reinfection: A 12-mo follow-up study. *World J Diabetes* 2022; 13(7): 543-552

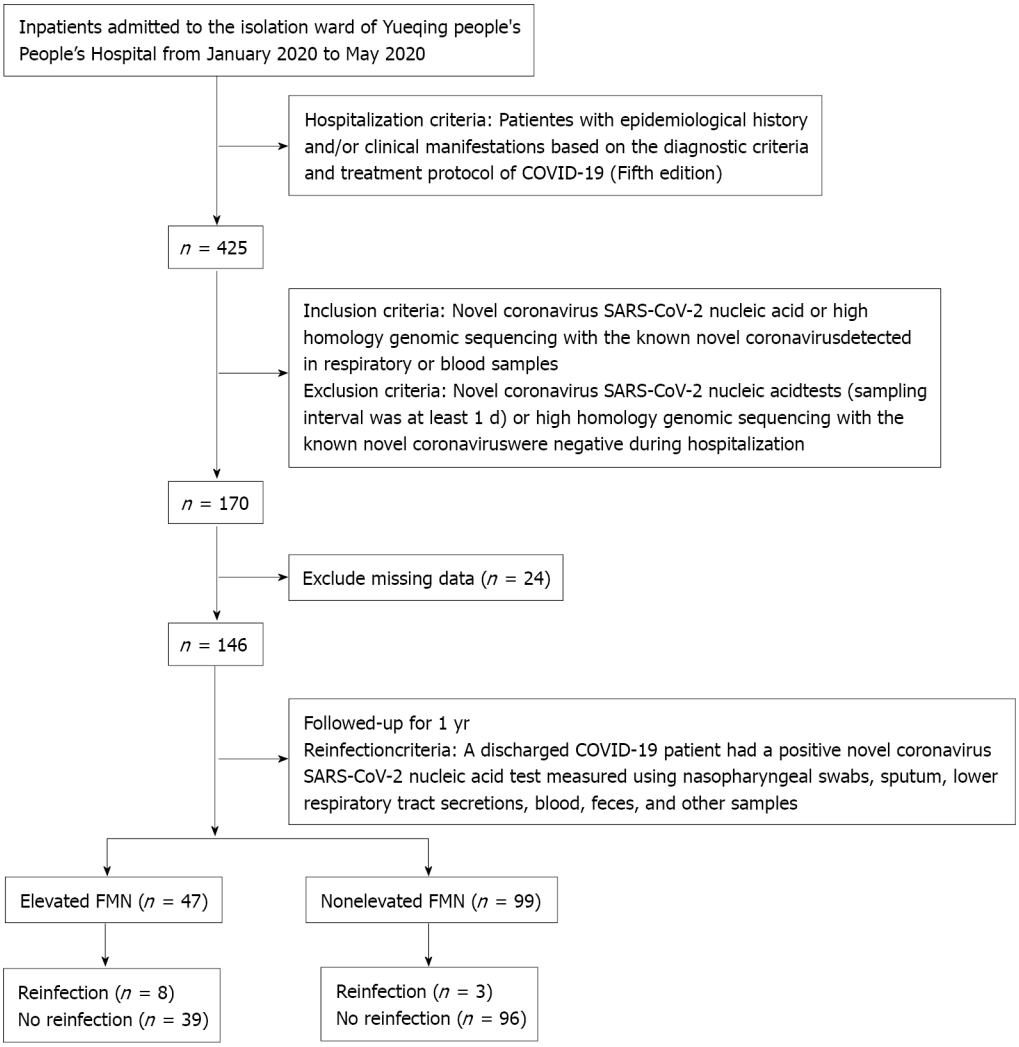
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/543.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i7.543

INTRODUCTION

Coronavirus disease 2019 (COVID-19) was identified as an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019. It is mainly transmitted by droplets, contact, and aerosols in confined spaces[1,2]. It is highly infectious and widespread[3,4], with more than 505 million patients infected globally, with a cumulative mortality rate of 1.2%[5]. Diabetes is a risk factor for COVID-19, which results in increased severity and mortality[6-8]. A previous study found that of the 570 patients who died or were discharged from hospital, the mortality rate was 6.2% (of 386) for patients without diabetes or hyperglycemia, compared to 28.8% (of 184) for patients who had diabetes and/or uncontrolled hyperglycemia[9]. Hyperglycemia is considered a factor for severity of infection, including severe pneumonia, multiple organ failure, and death. In addition, hemoglobin (Hb)A1c level is an independent risk factor for death and a predictor of COVID-19 severity in patients with diabetes mellitus[10,11].

Fructosamine (FMN) reflects the overall glycemic control for the past 2-3 wk[12] and is strongly correlated with glucose and HbA1c levels[13,14]. HbA1c reflects overall glycemic control over the past 2-3 mo, and general blood glucose monitoring reflects glucose levels at the point. General blood glucose monitoring and HbA1c levels cannot accurately contribute to a prediction index for recent glycemic control. FMN level can be determined rapidly and better reflects recent glycemic control. It has also been associated with diabetic retinopathy, diabetic nephropathy, and long-term cardiovascular outcomes[15]. In addition, FMN levels are positively associated with the risk of periprosthetic joint infection and negatively associated with cancer risk. A previous study also demonstrated that FMN is a valuable marker for predicting adverse outcomes following total hip arthroplasty[16]. Hence, FMN correlates with diabetic complications, inflammation, and cancer. However, to date, no studies have



DOI: 10.4239/wjd.v13.i7.543

Figure 1 Flowchart of the study cohort. FMN: Fructosamine; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

demonstrated its association with COVID-19 risk or SARS-CoV-2 reinfection. The objective of the present study was to determine whether there is an association between FMN levels and COVID-19 risk and SARS-CoV-2 reinfection. This may provide a theoretical basis for the clinical treatment and prognosis of SARS-CoV-2 reinfection.

MATERIALS AND METHODS

Study cohort

Between January and May 2020, we enrolled 146 patients from the isolation ward of Yueqing People's Hospital, a designated isolation hospital for COVID-19. All the patients met the diagnostic criteria and treatment protocol for COVID-19 (5th edition). Elevated FMN was defined as levels higher than the upper tertile value of 1.93 mmol/L. The study cohort was divided into two groups based on FMN levels (Figure 1), *i.e.*, elevated FMN group (> 1.93 mmol/L; n = 47) and nonelevated FMN group (≤ 1.93 mmol/L; n = 99). All patients were followed from January 2020 to May 2021, with an average follow-up period of 1 year. The study protocol was approved by the Ethics Committee of Yueqing People's Hospital, Affiliated Hospital of Wenzhou Medical University (No. YQYY202100033).

Laboratory measurements

Venous blood samples were collected after an overnight fast of ≥ 8 h. All laboratory data were obtained from the first serum collection during hospitalization. The absolute value of peripheral white blood cells (WBCs), lymphocytes, serum creatinine, liver function indexes (alanine and aspartate aminotransferases), lipid profiles (total cholesterol, triacylglycerol, high-density lipoprotein cholesterol, and low-

density lipoprotein cholesterol), and albumin were measured using standard methods. FMN levels were measured using the Roche automatic biochemical analyzer (Basel, Switzerland) and high performance liquid chromatography (Roche). The reference range for FMN was 1.15-2.25 mmol/L. Prognostic nutritional index (PNI) reflects the immune-nutritional status of patients and was determined by calculating serum albumin levels plus a fivefold total number of lymphocytes. PNI is associated with various cancers, such as lung, breast, and gynecological cancers[17].

Diagnostic criteria

The patients were diagnosed according to the Chinese Diagnostic Criteria and Treatment Protocol for COVID-19 (5th edition)[18].

Suspected cases: The patients were suspected to have COVID-19 based on a comprehensive analysis in combination with epidemiological history and clinical manifestations. The epidemiological history included: History of travel or residence in Wuhan and surrounding areas, or other communities where cases have been reported within 14 d before onset of illness; history of contact with a SARS-CoV-2-infected patient (positive for nucleic acid test) within 14 d before onset of illness; history of contact with patients with fever or respiratory symptoms from Wuhan and surrounding areas, or from communities where cases have been reported, within 14 d before onset of illness; and aggregation onset. Clinical manifestations included: Fever and/or respiratory symptoms; imaging features of SARS-CoV-2 pneumonia; normal or reduced total WBC count, or reduced lymphocyte count during the early stages of the disease. An individual with an epidemiological history and any two of the clinical manifestations were regarded as a suspected case. If there was no clear epidemiological history, three of the clinical manifestations should be satisfied.

Confirmed cases: Suspected cases with one of the following two tests being positive were regarded as confirmed cases: SARS-CoV-2 nucleic acid detected by real-time reverse transcription polymerase chain reaction in respiratory tract specimens or blood samples, and genomic sequencing of the respiratory or blood samples showing high homology with SARS-CoV-2.

Discharge criteria

A patient was discharged from isolation and transferred to other wards if his/her body temperature returned to normal and was stable for 3 d, respiratory symptoms improved significantly, lung imaging showed obvious improvement, and two nucleic acid tests were negative (sampling interval was at least 1 d).

Reinfection criteria

SARS-CoV-2 reinfection was defined when a discharged patient had a positive result on the SARS-CoV-2 nucleic acid test measured using nasopharyngeal swabs, sputum, lower respiratory tract secretions, blood, feces, and other samples.

Statistical analysis

IBM SPSS Statistics version 26 (IBM, Armonk, NY, United States) was used for statistical analyses. Normality of data distribution was determined by one-sample Kolmogorov-Smirnov test. Normally distributed data are expressed as the mean ± SD, and were determined using an independent group *t*-test. Non-normally distributed data are expressed as the median and interquartile range and were analyzed using the Mann-Whitney *U* test. The c^2 test was used for intergroup comparisons of categorical variables. Cox regression was used to determine the hazard ratio (HR) with 95% confidence interval (CI) for the positive reinfection across the tertiles of FMN levels, with the bottom tertile group as a reference. Kaplan-Meier analysis was used to determine the cumulative survival rate in patients with an FMN level higher than the top tertile compared with that in patients with nonelevated levels, tested using log-rank test. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study cohort

Of the 146 patients with COVID-19, 72 were male (49%) and 74 were female (51%), with an average age of 49 years. Comparison between the nonelevated FMN and elevated FMN groups showed no significant difference in gender, respiratory failure, WBC count, C-reactive protein, PNI, alanine transferase, aspartate aminotransferase, serum creatinine, triglyceride, total cholesterol, high-density lipoprotein, or low-density lipoprotein ($P > 0.05$) (Table 1). The average age of patients with elevated FMN was higher than that of patients in the nonelevated FMN group [53 (43-58) years *vs* 47 (35-53) years, $P = 0.008$] (Table 1). There were significant differences in diabetes mellitus, hypertension, and corticosteroid therapy between the two groups ($P < 0.05$) (Table 1).

Table 1 Baseline characteristics of the study cohort

Variable	Total	Elevated FMN[1]	Nonelevated FMN[2]	*P* value
Patients, *n* (%)	146	47 (68)	99 (32)	
Gender, *n* (%)				0.319
Male	72 (49)	26 (36)	46 (64)	
Female	74 (51)	21 (28)	53 (72)	
Age (yr)	49 (39-55)	53 (43-58)	47 (35-53)	0.008
Diabetes mellitus, *n* (%)	17 (12)	14 (82)	3 (18)	0.000
Hypertension, *n* (%)	18 (12)	8 (44)	10 (56)	0.023
Respiratory failure, *n* (%)	12 (8)	6 (50)	6 (50)	0.291
Corticosteroid therapy, *n* (%)	30 (21)	5 (17)	25 (83)	0.041
WBC [(4.0 × 10^9/L)-(10.0 × 10^9/L)]	4.64 (3.63-5.82)	5.06 (3.85-6.45)	4.58 (3.45-5.40)	0.067
CRP (< 5 mg/L)	7.30 (5.0-25.60)	6.80 (5.0-34.90)	7.80 (5.0-23.60)	0.320
PNI	47.80 (44.26-50.58)	49.55 (46.05-50.95)	47.05 (44.05-49.55)	0.061
ALT (0-55 U/L)	20.50 (14.0-29.0)	22.00 (15.0-31.0)	19.00 (13.0-28.0)	0.138
AST (0-55 U/L)	23.00 (18.0-31.0)	25.00 (19.0-32.0)	22.00 (18.0-30.0)	0.016
SCR (45-84 μmol/L)	62 (50-74)	64 (55-73)	61 (50-75)	0.460
TC (3.60-5.70 mmol/L)	4.24 ± 0.77	4.11 ± 0.79	4.30 ± 0.76	0.176
TG (0.60-1.70 mmol/L)	1.16 (0.86-1.69)	1.22 (0.88-1.77)	1.14 (0.86-1.66)	0.239
HDL-C (1.09-2.27 mmol/L)	0.95 (0.80-1.16)	0.92 (0.76-1.16)	0.98 (0.83-1.16)	0.314
LDL-C (1.30-3.37 mmol/L)	2.30 (1.94-2.91)	2.22 (1.90-2.78)	2.32 (1.98-2.92)	0.242
Reinfection case, *n* (%)	11 (7.5)	8 (73)	3 (17)	0.008

[1]Upper third of fructosamine levels.
[2]Lower two-thirds of fructosamine levels.
Data are presented as the mean (SD) for normally distributed data and median (interquartile range) for non-normal distributed data. PNI = serum albumin (g/L) + 5 × lymphocyte count (× 10^9/L). *P* value was calculated using one-sample Kolmogorov-Smirnov test or *t*-test. WBC: White blood cell count; FMN: Fructosamine; CRP: C-reactive protein; PNI: Prognostic nutritional index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; SCR: Serum creatinine; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Association of FMN levels with SARS-CoV-2 reinfection

The SARS-CoV-2 reinfection rate was significantly higher in patients in the elevated FMN group than in those in the nonelevated FMN group (17% *vs* 3%, $P = 0.008$) (Table 1). In the Cox regression model, disease-free survival (DFS) was used as the time variable, and reinfection was used as the state variable. After full adjustment, the elevated FMN group showed an increased risk of reinfection (HR = 6.249, 95%CI: 1.377-28.351, $P = 0.018$; P for trend < 0.05) (Table 2).

Association of FMN with cumulative DFS rate

Kaplan-Meier survival analysis showed that the cumulative DFS rate in the elevated FMN group was lower compared to that of the nonelevated FMN group (83% *vs* 97%, $P = 0.002$) (Figure 2). The survival rate was determined using the log-rank test, and the P for trend was < 0.05.

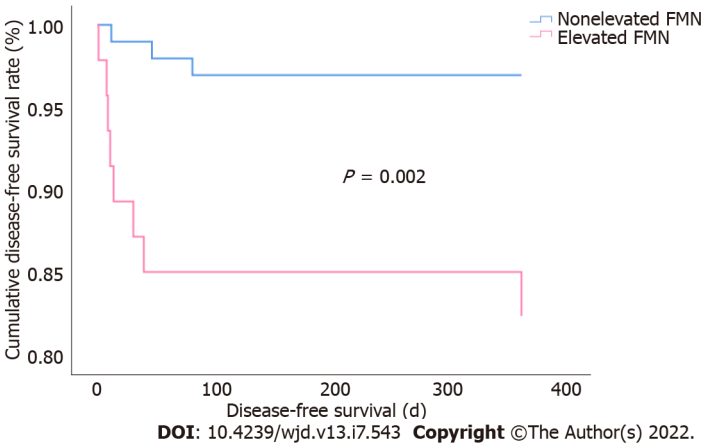
DISCUSSION

We found that patients with elevated FMN levels were older compared to patients in the nonelevated FMN group. Elevated FMN levels were positively associated with reinfection rate as well as HR for reinfection, while the cumulative DFS rate was lower in patients in the elevated FMN group. These results demonstrate that FMN levels may influence the prognosis of patients with COVID-19. COVID-19 is an acute inflammatory disease. Previous studies have demonstrated that patients with diabetes and severe disease were less likely to experience recurrence of SARS-CoV-2 infection[19]; however, patients with uncontrolled diabetes had an increased risk of reinfection[20]. Blood glucose monitoring reflects

Table 2 Association of fructosamine levels with SARS-CoV-2 reinfection

FMN dichotomy	B	SE	HR	95%CI	*P* value
Model 1	1.827	0.677	6.214	1.647-23.438	0.007
Model 2	1.898	0.759	6.674	1.507-29.544	0.012
Model 3	1.832	0.772	6.249	1.377-28.351	0.018

Model 1: Unadjusted. Model 2: Adjusted for age, gender, diabetes mellitus, corticosteroid therapy, and hypertension. Model 3: Adjusted for Model 2 and acute liver failure, acute kidney failure, white blood cell count, C-reactive protein, prognostic nutritional index, and blood lipids. *P* value for hazard ratio with 95% confidence interval was calculated using Cox regression models to indicate a significant association. FMN: Fructosamine; HR: Hazard ratio; CI: Confidence interval.



DOI: 10.4239/wjd.v13.i7.543

Figure 2 Association of fructosamine levels with cumulative disease-free survival rate. FMN: Fructosamine.

glucose levels at the point of testing and does not reflect overall blood glucose control. Compared to HbA1c, FMN can reflect blood glucose changes more recently. Previous studies have demonstrated that FMN is a good predictor of adverse events following total knee arthroplasty. Patients with high FMN levels were more likely to develop prosthetic joint infections compared to patients with low FMN levels. Unlike FMN, HbA1c does not show a significant association with complications[6]. FMN but not HbA1c is a significant predictor of infection in hemodialysis and diabetes patients with acute infections[21]. In our study, we found that FMN was associated with SARS-CoV-2 reinfection. Compared to patients with low FMN levels, patients with high FMN levels were found to have a higher reinfection rate. Patients with high FMN levels had a higher HR for reinfection, while patients with low FMN levels had higher cumulative DFS rates. It appears that FMN levels may predispose individuals to reinfection. Thus, the clinical focus should be on maintaining consistent euglycemia, using standard point-of-care glucose checks.

FMNs are advanced glycation end products (AGEs) generated when glucose reacts reversibly with amino groups in proteins. Reversible aldehyde imine intermediate is formed by the aldehyde group of carbohydrates and the N-terminal amino acids of proteins. However, irreversible AGEs are generated through a Maillard reaction[22]. Maillard reactions have been shown to impair cellular function[23]. FMN-3 kinase-related protein, designated as a potential longevity protein[24], can catalyze deglycation of Maillard intermediates directly downstream from FMN, thereby reducing AGE levels[25,26]. Several studies have demonstrated that AGE levels increase with age[27]. In our study, we found that older patients had higher FMN levels.

High FMN levels usually reflect hyperglycemia, which may lead to poor outcomes. Hyperglycemia enhances the expression of angiotensin-converting enzyme (ACE)2, which is the major cell entry receptor for SARS-CoV-2. ACE2 is widely expressed in the kidneys, lungs, and intestinal mucosal cells. SARS-CoV-2 can replicate abundantly in these sites and may contribute to reinfection[28] (Figure 3). Physiologically, hyperglycemia leads to a significant decrease in lymphocyte count, *i.e.*, $CD3^+$ and $CD4^+$ T cells, which in turn reduces humoral immunity mediated by macrophages and dendritic cells, and induces interleukin-6, tumor necrosis factor α, *etc.* to induce a cytokine storm[29]. This immunological disorder may increase the occurrence of antibody-dependent enhancement (ADE). In patients who are positive for coronavirus-specific antibodies or are infected by different virus strains, their antibodies may not neutralize the infection, but instead trigger FCγ receptor-mediated uptake of the virus, leading to an increase in virus numbers in the body[30,31] (Figure 4). Hence, ADE may be another pathological

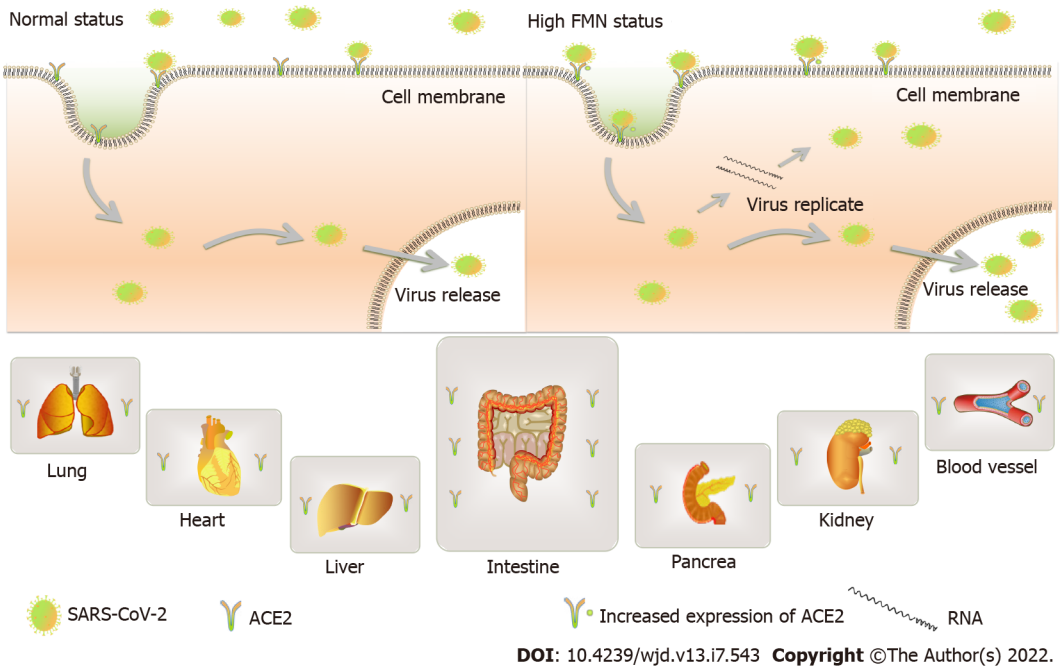


Figure 3 Potential pathways for reinfection in patients with high fructosamine levels and increased angiotensin-converting enzyme 2 expression. FMN: Fructosamine; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

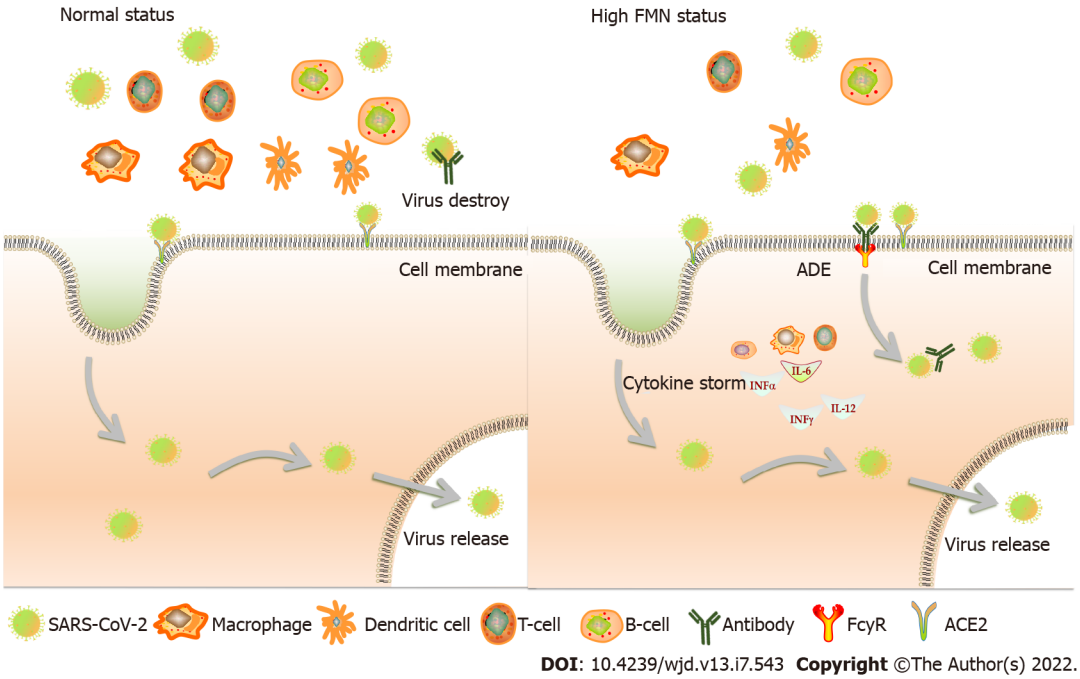


Figure 4 Potential pathways of reinfection in patients with high fructosamine levels with immunological disorders. FMN: Fructosamine; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; INF: Interferon; IL: Interleukin; ADE: Antibody-dependent enhancement.

mechanism of positive securement of SARS-CoV-2.

There were some limitations to the present study, which may have introduced potential bias. First, the study was a prospective, single center, small cohort study. Additional multicenter studies using larger patient cohorts should be performed to validate our findings. Second, HbA1c data for some of the patients were not available, which affected our comparative analysis of HbA1c and FMN levels. Third, diabetes was not excluded in the inclusion criteria, but we adjusted for diabetes.

CONCLUSION

Elevated FMN levels were found to predispose COVID-19 patients to reinfection and hence should be followed closely to monitor reinfection.

ARTICLE HIGHLIGHTS

Research background

Diabetes is a risk factor for coronavirus disease 2019 (COVID-19) which results in increased severity and mortality but has no relationship with COVID-19 reinfection. No study has reported the relationship between COVID-19 reinfection and blood levels of fructosamine (FMN). The present study for the first time reported this relationship.

Research motivation

We mainly investigate the relationship between blood levels of FMN and COVID-19 reinfection.

Research objectives

We found that FMN levels may influence the prognosis of patients infected with COVID-19, which highlight that the hospitalization patients with elevated levels of FMN should be cautiously monitored at post discharge.

Research methods

A total of 146 inpatients from the designated isolation hospital for COVID-19 patients, who were satisfied based on the diagnostic criteria and treatment protocol of COVID-19 (Fifth edition). The study cohort was divided into two groups based on FMN levels, elevated FMN was defined as levels higher than its upper tertile value, with the average follow-up period being one year. Cox regression was used to determine the hazard ratios (HRs) with 95% confidence intervals for the positive reinfection across the tertiles of FMN levels. Kaplan-Meier analysis was used to determine the cumulative survival rate in the patients with higher than the top tertiles of FMN levels compared with those with non-elevated levels, tested using log-rank.

Research results

We found that patients with elevated FMN levels were older than the non-elevated FMN group. Elevated FMN levels were positively associated with reinfection rate as well as HR for reinfection, while the cumulative disease-free survival rate was lower for patients in the elevated FMN group. These results demonstrate that FMN levels may influence the prognosis of patients infected with COVID-19.

Research conclusions

Elevated levels of FMN are independently associated with COVID-19 reinfection, which highlight that the COVID-19 patients with elevated levels of FMN should be followed up closely to monitor reinfection.

Research perspectives

Additional multicenter, hemoglobin A1c data available studies using larger patient cohorts should be performed to validate our findings.

ACKNOWLEDGEMENTS

The authors would like to thank Yueqing People's Hospital, Affiliated Hospital of Wenzhou Medical University for the data support and Dr. Bo Yang for his careful statistical assistance.

FOOTNOTES

Author contributions: Gu XJ was the guarantor and designed the study; Huang XY and Hu X participated in the acquisition, analysis, interpretation of the data, and drafted the initial manuscript; Yang LY, Zhang XX, Gu X, Du LJ, and He ZY revised the article critically for important intellectual content.

Institutional review board statement: The study protocol was approved by the Ethics Committee of Yueqing People's Hospital, Affiliated Hospital of Wenzhou Medical University (No. YQYY202100033).

Informed consent statement: The informed consent statement was waived.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: China

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S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 553-565

DOI: 10.4239/wjd.v13.i7.553

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Observational Study

Factors associated with trabecular bone score in postmenopausal women with type 2 diabetes and normal bone mineral density

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Bhattacharya S, India; Jones AR, Australia; Koo M, Taiwan

Received: February 15, 2022
Peer-review started: February 15, 2022
First decision: April 17, 2022
Revised: May 2, 2022
Accepted: June 24, 2022
Article in press: June 24, 2022
Published online: July 15, 2022

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Abstract

BACKGROUND

Osteoporosis and type 2 diabetes (T2D) have been recognized as a widespread comorbidity leading to excess mortality and an enormous healthcare burden. In T2D, bone mineral density (BMD) may underestimate the risk of low-energy fractures as bone quality is reduced. It was hypothesized that a decrease in the trabecular bone score (TBS), a parameter assessing bone microarchitecture, may be an early marker of impaired bone health in women with T2D.

AIM

To identify clinical and body composition parameters that affect TBS in postmenopausal women with T2D and normal BMD.

METHODS

A non-interventional cross-sectional comparative study was conducted. Potentially eligible subjects were screened at tertiary referral center. Postmenopausal women with T2D, aged 50-75 years, with no established risk factors for secondary osteoporosis, were included. BMD, TBS and body composition parameters were assessed by dual-energy X-ray absorptiometry. In women with normal BMD, a wide range of anthropometric, general and diabetes-related clinical and laboratory parameters were evaluated as risk factors for TBS decrease using univariate and multivariate regression analysis and analysis of receiver operating characteristic (ROC) curves.

RESULTS

Three hundred twelve women were initially screened, 176 of them met the inclusion criteria and underwent dual X-ray absorptiometry. Those with reduced BMD were subsequently excluded; 96 women with normal BMD were included in final analysis. Among them, 43 women (44.8%) showed decreased TBS values (≤

1.31). Women with TBS ≤ 1.31 were taller and had a lower body mass index (BMI) when compared to those with normal TBS (P = 0.008 and P = 0.007 respectively). No significant differences in HbA1c, renal function, calcium, phosphorus, alkaline phosphatase, PTH and 25(OH)D levels were found. In a model of multivariate linear regression analysis, TBS was positively associated with gynoid fat mass, whereas the height and androgen fat mass were associated negatively (all P < 0.001). In a multiple logistic regression, TBS ≤ 1.31 was associated with lower gynoid fat mass (adjusted odd ratio [OR], 0.9, 95% confidence interval [CI], 0.85-0.94, P < 0.001), higher android fat mass (adjusted OR, 1.13, 95%CI, 1.03-1.24, P = 0.008) and height (adjusted OR, 1.13, 95%CI, 1.05-1.20, P < 0.001). In ROC-curve analysis, height ≥ 162.5 cm (P = 0.04), body mass index ≤ 33.85 kg/m^2 (P = 0.002), gynoid fat mass ≤ 5.41 kg (P = 0.03) and android/gynoid fat mass ratio ≥ 1.145 (P < 0.001) were identified as the risk factors for TBS reduction.

CONCLUSION

In postmenopausal women with T2D and normal BMD, greater height and central adiposity are associated with impaired bone microarchitecture.

Key Words: Diabetes; Osteoporosis; Bone mineral density; Trabecular bone score; Obesity; Body composition

Core Tip: In this study, we assessed the impact of a wide range of general and diabetes-related parameters on trabecular bone score (TBS) in postmenopausal women with type 2 diabetes (T2D) and normal bone mineral density (BMD). A decrease in TBS was revealed in 44.8% of study participants. These data indicate that a substantial proportion of postmenopausal women with T2D and normal BMD may have impaired bone microarchitecture. Greater height and central adiposity turned out to be the risk factors for decreased TBS in these women.

Citation: Fazullina ON, Korbut AI, Klimontov VV. Factors associated with trabecular bone score in postmenopausal women with type 2 diabetes and normal bone mineral density. *World J Diabetes* 2022; 13(7): 553-565
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/553.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.553

INTRODUCTION

Type 2 diabetes (T2D) and bone fractures have been recognized as a widespread comorbidity leading to excess mortality and an enormous healthcare burden[1,2]. Recent data from the Continuous National Health and Nutrition Examination Survey (NHANES) indicate an increasing prevalence of osteoporosis and osteopenia in the US among T2D patients[3]. People with T2D have higher risk of vertebral and some non-vertebral fractures than non-diabetic individuals[4,5], regardless of normal or even increased bone mineral density (BMD)[6,7]. This "diabetic paradox" has been attributed to the modified effect of hyperglycemia, obesity and related factors on BMD[8]. As BMD assessment may lead to underestimation of a fracture risk in T2D, additional parameters of bone health should be taken into consideration.

In recent years, the Trabecular Bone Score (TBS) on lumbar spine dual X-ray absorptiometry (DXA) images is increasingly applied for the assessment of bone microarchitecture. It had been demonstrated that low TBS is associated with both prevalent and incident fractures; therefore, TBS was incorporated in the Fracture Risk Assessment tool (FRAX) algorithm[9]. The impaired bone microarchitecture is considered as a major contributor to fracture risk in T2D[10]. Accordingly, the utility of TBS for osteoporotic fracture risk assessment was shown in postmenopausal women with T2D[11,12]. Individuals with diabetes as compared to those without have significantly lower TBS[13,14]; the difference is greater in women[13]. It could be speculated that the reduction of TBS is an earlier event in the deterioration of bone health in T2D than BMD decrease. However, at present, data on TBS in postmenopausal women with T2D and normal BMD is limited, and predictors of the TBS decrease in these women need to be refined.

A growing body of evidence indicates the pivotal role of hyperglycemia-related biochemical abnormalities, as well as obesity and dysregulated adipokine production, in the pathogenesis of increased bone fragility in T2D[15,16]. Nevertheless, the role of diabetes-related factors and fat accumu-

lation at early stages of bone metabolic disease in T2D needs further research.

Therefore, in this study we aimed to identify clinical and body composition parameters that affect TBS in postmenopausal women with T2D and normal BMD.

MATERIALS AND METHODS

Design

A non-interventional cross-sectional comparative study was conducted.

To be included in the study, women had to meet the following criteria: (1) Caucasian origin; (2) Age 50-75 years; (3) Time since menopause ≥ 1 year; (4) Known T2D duration ≥ 1 year; and (5) Normal BMD assessed by DXA.

The following list of exclusion criteria was applied: Endocrine diseases other than T2D (hyperthyroidism, hypothyroidism, hyperparathyroidism, hypopituitarism, acromegaly, and Cushing syndrome); Rheumatic diseases (rheumatoid arthritis, psoriatic arthritis, spondyloarthritis, systemic lupus erythematosus, systemic sclerosis, vasculitis, and crystal-induced arthritis); Inflammatory bowel diseases, celiac disease, malabsorption or bariatric surgery in medical history; Chronic kidney disease with an estimated glomerular filtration rate (eGFR) less than 45 mL/min/1.73 m^2; Ever diagnosed with any kind of malignancy; Immobilization for more than one month in medical history; Treatment with thiazolidinediones, glucocorticoids, anticonvulsants or immunosuppressive drugs, postmenopausal hormonal replacement therapy, anti-osteoporotic therapy at the time of the study or in the past.

Potentially eligible subjects were screened at the clinic of Research Institute of Clinical and Experimental Lymphology - Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences (Novosibirsk, Russa), a tertiary referral center. All women underwent a detailed clinical examination, which included the assessment of glycemic control, in-depth screening/monitoring of diabetic complications and associated diseases. Women who met the inclusion criteria (1-4) and did not have the exclusion criteria underwent DXA to determine body composition, BMD and TBS. Those with abnormal BMD (T-score ≤ -1 SD) were excluded. The rest of the participants were divided into 2 groups: 1) women with normal TBS (>1.31); 2) women with TBS reduction (≤1.31). The cut-off TBS value was chosen according to the results of meta-analysis [17]. The risk factors for TBS reduction were estimated by univariate and multivariate regression analysis and analysis of receiver operating characteristic (ROC)-curves.

Ethical issues

The study protocol was approved by the Ethical Committee of the clinic of Research Institute of Clinical and Experimental Lymphology - Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences (protocol N. 104 from 20 December 2014). All study participants provided informed written consent prior to study enrollment.

Methods

DXA and fracture risk assessment: The BMD and T-score at the lumbar spine (L1–L4), femur, femoral neck and forearm were assessed by DXA (Lunar Prodigy Advance bone densitometer, GE healthcare, Madison, WI, United States; database NHANES III; the Least significant change is 0.028 g/cm^2 for L1-L4, 0.033 g/cm^2 for femur, and 0.055 g/cm^2 for radius 33%). The TBS was estimated with the use of TBS iNsight software (version 3.0.2.0, GE healthcare). The Body Composition software (GE healthcare) was applied for assessment of body composition parameters, including bone mineral component, fat mass and lean mass, and fat distribution. Fat distribution patterns were differentiated based on the ratio of fat mass in the abdominal and hip areas (android and gynoid fat mass respectively)[18].

The FRAX tool (web version 4.3, country-specific algorithm, https://www.sheffield.ac.uk/FRAX/tool.aspx?country=13) was used to determine the ten-year risk of low-energy fractures. Both TBS-unadjusted and TBS-adjusted FRAX scores were calculated.

Laboratory investigations: The measurements of the levels of glycated hemoglobin A1c (HbA1c), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, uric acid, creatinine, calcium, phosphorus and alkaline phosphatase were performed with a biochemical analyzer AU480 (Beckman Coulter, Minneapolis, MN, United States). eGFR was calculated using the CKD-EPI formula (2009). Albumin concentrations were determined in the morning urine samples by immunoturbidimetric method with a fully automated chemistry analyzer BS-120 (Mindray, Shenzhen, China); the result was adjusted to excreted creatinine. Serum levels of PTH and 25(OH)D were measured by ELISA with the use of Access 2 Immunoassay System analyzer (Beckman Coulter) and Access Intact PHT, Access 25(OH) Vitamin D Total kits (Beckman Coulter).

Statistical analysis

Dell Statistica 13.0 (Dell Software, Aliso Viejo, CA, United States) was used for most of the applied statistical procedures. The sample size was calculated with a predetermined Type I error rate $\alpha = 0.05$,

power goal 1–β = 80% and standardized size effect 0.5 for clinical characteristics (age, duration of diabetes, age and duration of menopause, height, body weight, body mass index [BMI], waist-to-hip circumference), laboratory parameters (HbA1c, eGFR, calcium, phosphorus, 25(OH)D, PTH) and body composition (fat and lean mass, android and gynoid fat mass and percentage, android/gynoid fat mass ratio). The minimal number of participants in each group was defined as 34 persons. Assuming the prevalence of osteoporosis[19,20] and decreased TBS[21,22] in patients with T2D and using principles described previously[23,24], we estimated the minimal number of study participants as 150 individuals.

Quantitative data are presented as medians (lower quartiles; upper quartiles), frequencies are presented as percentages (%). The Kolmogorov–Smirnov (KS) test was applied to test the normality. As the majority of the quantitative parameters were not distributed normally, the non-parametric Mann–Whitney U-test was used for the group comparisons. The differences in discrete parameters were assessed using the χ^2 test. *P* values below 0.05 were considered as significant.

Spearman rank correlation analysis was applied to test associations between variables. Multiple linear regression analysis with backward elimination was used to reveal factors affecting TBS. The description of the model included beta coefficients with standard errors and *P* values, adjusted coefficient of determination (R^2), standard error of estimate and *P* value of the model.

Multiple logistic regression analysis with backward elimination was used to identify predictors of decreased TBS. The models with lower KS statistics p value and higher area under the curve (AUC), selectivity (Se), and specificity (Sp) were selected. Crude and adjusted odd ratio (OR), 95% confidence interval (CI) and *P* value were calculated for parameters included in the models.

To assess the parameters associated with decreased TBS, ROC-curve analysis was performed with IBM SPSS Statistics for Windows, Version 26.0 (International Business Machines Corporation, Armonk, NY, USA). The AUC with 95%CI and *P* value were calculated. The results were considered significant if the AUC with a lower border of 95%CI was above 0.5 and *P* value was below 0.05. The cut-off values were found with both Se and Sp above 0.55.

RESULTS

Study participants

Three hundred twelve women were initially screened, 176 of them met the inclusion criteria (1-4). These subjects underwent DXA with BMD and TBS assessment. According to DXA results, 17 women had osteoporosis and 63 had osteopenia; these individuals were excluded. Ultimately, 96 women with normal BMD were included in the final analysis.

The mean age of women was 64 years (range: 50-75 years) and mean time since menopause was 16 years (range: 1-37 years). Thirteen women were overweight, 79 were obese and four had a normal BMI. The BMI ranged from 19.1 to 50.2 kg/m^2 (median 33.6 kg/m^2). The duration of T2D varied from 1 to 48 years (median 15 years). All patients received antihyperglycemic therapy, including metformin (n = 80), sulfonylurea (n = 34), sodium glucose cotransporter 2 inhibitors (n = 26), dipeptidyl peptidase-4 inhibitors (n = 9), and insulin (n = 70), mostly in combinations. The mean level of HbA1c was 8.76% (72.2 mmol/mol), ranging from 5.61 to 13.64% (37.7 to 125.6 mmol/mol).

Characteristics of women with T2D depending on TBS values

The clinical characteristics of women with preserved and decreased TBS are presented in Table 1. Women with TBS ≤ 1.31 were taller and had a lower BMI when compared to those with normal TBS (*P* = 0.008 and *P* = 0.007 respectively). There was a trend towards greater age and longer diabetes duration in women with TBS ≤ 1.31 (*P* = 0.09 and *P* = 0.052 respectively). The levels of HbA1c were slightly higher in women with TBS ≤ 1.31, but the difference with women with TBS > 1.31 were not statistically significant (*P* = 0.13). No differences in HbA1c, eGFR, calcium, phosphorus, alkaline phosphatase, PTH and 25(OH)D levels were found between the groups. Most women, including 45 (84.9%) with TBS > 1.31 and 38 women (88.4%) with TBS ≤ 1.31, had 25(OH)D concentrations < 30 ng/mL. The prevalence of diabetic complications and diabetes-associated conditions, as well as antihyperglycemic therapy, did not differ between the groups.

Six women with TBS > 1.31 and 14 women with TBS ≤ 1.31 had at least one fracture in their medical history (χ^2 = 5.64, *P* = 0.02). Two women with TBS > 1.31 had a low-energy fracture (humerus, tibia) in anamnesis. In the group of patients with TBS ≤ 1.31, nine women reported low-energy fractures of spine (n = 2), radius (n = 4), femur neck (n = 1) and humerus (n = 2). A difference in the prevalence of low-energy fractures was statistically significant (χ^2=6.05, *P* = 0.01). At the same time, there were no differences in BMD and T-score between two groups (Table 2). The 10-year risk of low-grade hip fractures was higher in those with TBS ≤ 1.31 (all *P* < 0.0001). The inclusion of TBS data in the FRAX algorithm exacerbated the differences between the groups.

Women with reduced TBS had lower gynoid fat mass and higher android/gynoid fat mass ratio (*P* = 0.004 and *P* < 0.0001 respectively). No differences in trunk fat mass, lean mass and BMC were found (Table 3).

Table 1 Clinical characteristics of postmenopausal women with type 2 diabetes depending on trabecular bone score values

Parameter	Women with TBS > 1.31 (*n* = 53)	Women with TBS ≤ 1.31 (*n* = 43)	*P* value
Age (yr)	62 (59; 68)	65 (59; 72)	0.09
Age at menopause (yr)	50 (46; 53)	50 (45; 52.5)	0.6
Time since menopause (yr)	14 (10; 19)	17 (9; 21.5)	0.72
Diabetes duration (yr)	14 (10; 20)	19 (12; 23)	0.052
Height (cm)	160 (156; 165)	164 (160; 167)	0.008
Body weight (kg)	90 (81; 101)	84 (80; 93)	0.2
BMI (kg/m^2)	35.3 (32.5; 37.2)	32 (29.7; 34.9)	0.007
WHR	0.95 (0.93; 1.0)	1.02 (0.9; 1.05)	0.37
HbA1c (%)	8.5 (7.1; 9.3)	8.9 (7.7; 10.1)	0.13
Total cholesterol (mmol/L)	4.4 (4.1; 5.6)	5.1 (3.9; 5.7)	0.44
LDL-cholesterol (mmol/L)	2.9 (2.6; 3.7)	3.4 (2.7; 3.9)	0.21
HDL-cholesterol (mmol/L)	1.3 (1.1; 1.5)	1.2 (1.1; 1.5)	0.67
Triglycerides (mmol/L)	1.8 (1.1; 2.5)	2.1 (1.4; 2.7)	0.31
hsCRP (mmol/L)	3.1 (1.8; 8.3)	3.3 (1.5; 7.3)	0.71
Calcium (mmol/L)	2.4 (2.4; 2.5)	2.4 (2.4; 2.5)	0.96
Phosphorus (mmol/L)	1.2 (1.1; 1.4)	1.3 (1.2; 1.4)	0.11
Alkaline phosphatase (IU/L)	84.6 (67.3; 107.3)	81.1 (64.8; 98.5)	0.66
PTH (pg/mL)	32.4 (24; 45.4)	31.2 (15.3; 39.0)	0.36
25(OH)D (ng/mL)	21.3 (15.8; 26.5)	18.7 (12.4; 24.2)	0.07
eGFR (mL/min/1.73 m^2)	76 (55; 93)	72 (57; 92)	0.7
UACR (mg/mmoL)	0.6 (0.3; 1.1)	0.5 (0.3; 1.0)	0.18
Diabetic retinopathy, *n* (%)	24 (45.3%)	24 (55.8%)	0.38
CKD, *n* (%)	22 (41.5%)	20 (46.5%)	0.89
Diabetic neuropathy, *n* (%)	53 (100%)	43 (100%)	0.76
Peripheral artery disease, *n* (%)	19 (35.8%)	19 (48.3%)	0.42
Coronary artery disease, *n* (%)	17 (32.1%)	11 (27.6%)	0.59
Metformin, *n* (%)	43 (81.1%)	37 (86%)	0.68
Sulfonylurea, *n* (%)	20 (37.7%)	14 (32.6%)	0.67
DPP4 inhibitor, *n* (%)	3 (5.7%)	6 (14%)	0.49
SGLT2 inhibitor, *n* (%)	16 (30.2%)	10 (23.3%)	0.56
Insulin, *n* (%)	38 (71.7%)	32 (74.4%)	0.82
Fracture in medical history, *n* (%)	6 (11.3%)	14 (32.6%)	0.02
Low-energy fracture in medical history, *n* (%)	2 (3.8%)	9 (20.9%)	0.01

Data are presented as medians (25; 75 percentiles). TBS: Trabecular bone score; BMI: Body mass index; WHR: Waist-to-hip ratio; HbA1c: Hemoglobin A1c; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; hsCRP: High-sensitivity C-reactive protein; PTH: Parathyroid hormone; 25(OH)D: 25-hydroxyvitamin D; eGFR: Estimated glomerular filtration rate; UACR: Urinary albumin-to-creatitine ratio; CKD: Chronic kidney disease; DPP4: Dipeptidyl peptidase-4; SGLT2: Sodium glucose cotransporter 2.

Associations of TBS with clinical and laboratory parameters

In observed women, TBS correlated positively with BMI (r = 0.33, P = 0.001), total fat mass (r = 0.26, P = 0.01) and gynoid fat mass (r = 0.39, P = 0.001). Height and android/gynoid fat mass ratio demonstrated inverse correlations with TBS (r = -0.26, P = 0.01 and r = -0.44, P = 0.00001 respectively), meanwhile, all assessed laboratory parameters, with the exception of 25(OH)D, did not show significant relationships.

Table 2 Dual X-ray absorptiometry parameters and Fracture Risk Assessment tool scores in postmenopausal women with type 2 diabetes depending on trabecular bone score values

Parameter	Women with TBS > 1.31 (*n* = 53)	Women with TBS ≤ 1.31 (*n* = 43)	*P* value
TBS	1.465 (1.39; 1.514)	1.206 (1.127; 1.271)	< 0.001
T-score, minimal	0.0 (-0.5; 0.5)	-0.2 (-0.6; 0.3)	0.42
T-score, L1-L4	0.9 (0.1; 1.7)	1.0 (0.1; 1.7)	0.84
T-score, femoral neck	0.1 (-0.2; 0.7)	-0.05 (-0.5; 0.5)	0.42
T-score, total femur	1.3 (0.85; 1.6)	1.3 (0.6; 1.8)	0.97
T-score, radius	0.3 (-0.3; 0.7)	-0.05 (-0.7; 0.7)	0.27
BMD, L1-L4 (g/cm^2)	1.278 (1.197; 1.387)	1.317 (1.205; 1.39)	0.86
BMD, neck (g/cm^2)	1.044 (1.011; 1.129)	1.045 (0.968; 1.105)	0.45
BMD, total femur (g/cm^2)	1.173 (1.099; 1.210)	1.178 (1.088; 1.237)	0.85
BMD, radius, (g/cm^2)	0.897 (0.849; 0.939)	0.873 (0.816; 0.938)	0.27
FRAX major (%)	6.1 (5.7; 6.8)	6.4 (5.7; 7.1)	0.38
FRAX hip (%)	0.1 (0.1; 0.3)	0.3 (0.1; 0.4)	0.08
FRAX major, TBS-adjusted (%)	5.1 (4.6; 6.0)	7.8 (6.9; 9.2)	< 0.001
FRAX hip, TBS-adjusted (%)	0.1 (0.0; 0.2)	0.4 (0.2; 0.6)	< 0.001

Data are presented as medians (25; 75 percentiles). TBS: Trabecular bone score; BMD: Bone mineral density; FRAX: The Fracture Risk Assessment Tool; FRAX hip: 10-year risk of hip low-energy fractures; FRAX major: 10-year risk of major low-energy fractures.

Table 3 Body composition parameters in postmenopausal women with type 2 diabetes depending on trabecular bone score values

Parameter	Women with TBS > 1.31 (*n* = 53)	Women with TBS ≤ 1.31 (*n* = 43)	*P* value
Total fat mass (%)	45.1 (41.7; 48.3)	43.7 (40.2; 46.2)	0.1
Total fat mass (kg)	40.4 (33.0; 40.4)	36.8 (32.4; 39.5)	0.12
Trunk fat mass (kg)	23.0 (18.8; 25.9)	21.9 (20.2; 25.1)	0.89
Android fat mass (kg)	4.0 (2.9; 4.6)	3.9 (3.5; 4.7)	0.47
Gynoid fat mass (kg)	5.8 (4.9; 6.9)	4.9 (4.3; 5.9)	0.004
Android/gynoid fat mass ratio	1.07 (0.99; 1.17)	1.18 (1.12; 1.29)	< 0.001
Lean mass (kg)	48.2 (44.4; 52.0)	47.7 (44.0; 52.1)	0.83
Bone mineral component (kg)	2.5 (2.4; 2.6)	2.5 (2.3; 2.7)	0.8

Data are presented as medians (25; 75 percentiles). TBS: trabecular bone score.

The levels of 25(OH)D demonstrated weak positive correlation with TBS (r = 0.21, P = 0.042). In addition, 25(OH)D correlated negatively with android fat mass (r = -0.20, P = 0.048), waist circumference (r = -0.24, P = 0.024), PTH (r = -0.34, P = 0.006), and alkaline phosphatase (r = -0.28, P = 0.007).

In a model of multivariate linear regression analysis, TBS was positively associated with gynoid fat mass (+0.007 per each 100 g), whereas the influence of height and androgen fat mass was negative (-0.008 per each cm and and -0.007 per each 100 g, respectively, Table 4). The same factors were identified in a multiple logistic regression model (Table 5). Thus, gynoid fat mass turned out to be a protective factor for TBS (-10% per each 100 g), while height and android fat mass were the risk factors for TBS reduction (+13% per each cm and each 100 g). However, the influence of android fat mass became significant only after being adjusted on height and gynoid fat mass. Moreover, the influence of all factors included in the logistic regression model increased after adjustment.

We have used ROC-analysis to estimate the cut-off values of the factors associated with TBS (Table 6). The height ≥ 162.5 cm, BMI ≤ 33.85 kg/m^2, gynoid fat mass ≤ 5.4 kg (≤ 43.2%), and android/gynoid fat mass ratio ≥ 1.15 were identified as the risk factors of decreased TBS.

Table 4 Factors associated with trabecular bone score in postmenopausal women with type 2 diabetes

Parameter	Coefficient β ± SE	*P* value
Height (cm)	-0.008 ± 0.002	< 0.001
Android fat (100 g)	-0.007 ± 0.002	< 0.001
Gynoid fat (100 g)	0.007 ± 0.002	< 0.001

The linear regression models with backward stepwise selection. Parameters of the model: Intercept 2.54 ± 0.39, adjusted R2 0.31, SE of estimate 0.14, *P* value < 0.001.

Table 5 Factors associated with decreased trabecular bone score in postmenopausal women with type 2 diabetes

Parameter	Crude OR, 95%CI, *P* value	Adjusted OR, 95%CI, *P* value
Height, cm	1.10 (1.02-1.19), *P* = 0.01	1.13 (1.03-1.24), *P* = 0.008
Android fat, 100 g	1.02 (0.98-1.05), *P* = 0.38	1.13 (1.05-1.20), *P* < 0.001
Gynoid fat, 100 g	0.96 (0.93-0.99), *P* = 0.01	0.90 (0.85-0.94), *P* < 0.001

The logistic regression models with forward stepwise selection. Parameters of the model: Intercept 19.0, Kolmogorov–Smirnov test *P* value < 0.001, area under the curve 0.82, Selectivity 0.74, Specificity 0.77, OR 7.69, 95%CI (3.08-19.2), *P* < 0.001 for cut-off value of logistic function = 0.47. 95%CI: 95% confidence interval; OR: Odd ratio.

Table 6 Risk factors of decreased trabecular bone score in postmenopausal women with type 2 diabetes estimated by receiver operating characteristic-analysis

Parameter	Cut-off points	Se	Sp	AUC ± SE (95%CI), *P* value	OR (95%CI), *P* value
Height (cm)	≥ 162.5	0.605	0.604	0.66 ± 0.06 (0.55-0.77), *P* = 0.009	2.33 (1.02-5.31), *P* = 0.04
BMI (kg/m^2)	≤ 33.85	0.70	0.62	0.66 ± 0.06 (0.55-0.77), *P* = 0.008	3.81 (1.62-8.96), *P* = 0.002
Gynoid fat (kg)	≤ 5.41	0.63	0.60	0.67 ± 0.06 (0.56-0.78), *P* = 0.004	2.49 (1.09-5.71), *P* = 0.03
Android fat mass (kg)	≥ 3.95	0.49	0.48	0.54 ± 0.06 (0.43-0.66), *P* = 0.46	0.88 (0.39-1.98), *P* = 0.76
Android/gynoid fat	≥ 1.145	0.70	0.71	0.75 ± 0.05 (0.66-0.85), *P* < 0.001	5.69 (2.35-13.79), *P* < 0.001

Sp: Specificity; Se: Sensitivity; AUC: Area under the curve; SE: Standard error; OR: Odd ratio; 95%CI: 95% Confidence interval; BMI: Body mass index.

DISCUSSION

In this study, we investigated the effects of a number of anthropometric parameters, general and diabetes-related clinical characteristics and body composition on bone microarchitecture, assessed by TBS, in postmenopausal women with T2D and normal BMD.

To date, several imaging modalities, including DXA, radiography, micro-computed tomography, high-resolution peripheral quantitative computed tomography (HR-pQCT), and high-resolution magnetic resonance imaging, have been proposed for bone quality assessment[25]. Among these methods, HR-pQCT and TBS are the most used tools to study the bone microarchitecture in diabetes [26]. HR-pQCT is a non-invasive three-dimensional imaging modality for assessment of bone microarchitecture and bone strength in the appendicular skeleton (*i.e.*, distal radius and tibia)[27]. In the Framingham-HR-pQCT study a modest deterioration in cortical bone and reductions in bone area in patients with T2D were revealed[28]. At the same time, in another population-based study by Nilsson *et al*[29] more favorable bone microarchitecture was observed in elderly women with T2D compared to non-diabetic subjects. TBS is a gray-level textural metric that can be extracted from the two-dimensional lumbar spine DXA image[30]. This analytical method for bone microarchitecture assessment is more available and less expensive than HRpQCT.

The normal range for TBS remains a matter of debate. In 2012, an international working group of TBS users proposed the following criteria: TBS ≥ 1.35 is considered to be normal; TBS between 1.20 and 1.35 indicates partially degraded microarchitecture; finally, TBS ≤ 1.20 defines degraded microarchitecture [31]. Later, based on the results of meta-analysis of 14 population cohort studies from North America, Asia, Australia, and Europe (n = 17809) estimated relationship between TBS and fracture risk, slightly

different criteria for assessing TBS have been proposed[17]. TBS > 1.31 was attributed to normal microarchitecture, TBS values between 1.23 and 1.31 were associated with partially degraded microarchitecture, and TBS < 1.23 was considered as an indicator of degraded microarchitecture. Taken into account that fractures are the most important clinical events related to the bone health, in this study we also used the cut-off value 1.31 to differentiate women with normal and degrade microarchitecture. This cut-off point has been also applied in recent osteoporosis studies[32,33]. Given the relatively small sample size, we did not distinguish a subgroup of patients with borderline TBS (1.23–1.31).

A significant proportion (44.8%) of women in our study showed TBS values less than 1.31. Earlier it was found that T2D women 50 years old and over had lower TBS but higher BMD when compared to non-diabetic women[11]. Postmenopausal women with newly diagnosed T2D showed a decrease in TBS and bone formation markers[34]. A recent study has demonstrated a negative association between TBS and pre-diabetes in subjects aged over 60 years and discordance between TBS and BMD in these subjects [35]. Therefore, the reduction of TBS may reflect an early stage of the impairment of bone health in diabetes. Previously an inverse association between age and TBS was observed in population studies in French and non-Hispanic white US women[36,37]. In this study we were unable to identify age as an independent risk factor for TBS reduction. This can be explained by the relatively small sample size, the upper age limit of 75 years, and the greater influence of other risk factors.

Our results indicate that greater height, lower BMI and gynoid fat mass, but higher android fat mass and android/gynoid fat mass ratio contribute to TBS decrease in women with T2D. A favorable effect of BMI and fat mass on BMD in postmenopausal women with T2D was documented in previous studies [38,39]. However, data on the effect of obesity on the bone metabolism, TBS and fracture risk are not so optimistic[40-42]. In disagreement with previously reported data[43], we observed a positive association between BMI and TBS. At the same time, we found negative association between android/gynoid fat mass ratio and TBS. Moreover, gynoid fat turned out to be a protective factor and android fat was a risk factor for TBS reduction. These findings provide further support to notion that not only fat mass, but also fat distribution, is important for bone health. Previously, inverse association between android fat and TBS was found in Chinese men[44]. Moon *et al*[40] have shown that TBS increase as visceral fat mass decrease in men and women with T2D. In the Newcastle Thousand Families Study an increase in total and, especially, visceral fat mass was associated with prevalent vertebral fracture irrespective of BMD in women aged about 62 years[41]. It was shown that abdominal fat is related to retarded bone formation and impaired bone quality in premenopausal women[42]. Therefore, central adiposity can be considered as a risk factor of bone fragility in T2D.

The association between abdominal obesity and impaired bone microarchitecture can be mediated *via* insulin resistance[43]. Increased bone marrow adiposity, the changes in adipokine production and low-grade inflammation are considered as the relevant mechanisms also[45]. In addition, vitamin D deficiency can worsen bone microarchitecture in women with T2D and abdominal obesity. In our cohort, 25(OH)D demonstrated negative correlation with waist circumference and abdominal fat mass. This data is in agreement with findings from recent meta-analysis of epidemiologic studies indicating an association between vitamin D deficiency and abdominal obesity[46]. Vitamin D deficiency in obese people is attributed to lower dietary intake of vitamin D, lesser skin exposure to sunlight, decreased vitamin absorption, impaired hydroxylation in adipose tissue and 25(OH)D accumulation in fat[47]. At the same time, it is believed that vitamin D deficiency can be associated with insulin resistance and related disorders[48,49].

The role of hyperglycemia as a factor contributing to the degradation of bone microarchitecture is widely discussed. The mechanisms of bone fragility in hyperglycemia include the accumulation of advanced glycation end products and collagen cross-linking, suppressed osteoid mineralization, reduced osteoblastogenesis, and retarded bone turnover[50]. Ho-Pham *et al*[13] reported that subjects with pre-diabetes have a decrease in TBS when compared with normal individuals. At the same time, Holloway *et al*[14] found no difference in TBS between subjects with normoglycaemia and impaired fasting glucose. A negative association between TBS and HbA1c has been reported in subjects with diabetes[51]. In the Maasticht study a negative association was found between HbA1c and parameters of bone health estimated by HR-pQCT in individuals with well-controlled T2D[52]. In our study, HbA1c was only slightly higher in patients with TBS ≤ 1.31. Even though we did not identify HbA1c as a risk factor for a decrease TBS, we cannot exclude the role of hyperglycemia in the deterioration of bone microarchitecture. Most of the patients had long-term diabetes and non-target glycemic control parameters on combined antidiabetic therapy. These factors could modify the effect of hyperglycemia on TBS. Besides, single HbA1c measurements were included in the analysis. Therefore, the effect of metabolic memory on bone structure cannot be ruled out.

The value of TBS as a predictor of low-energy fractures is a matter of increasing interest. It was demonstrated that in postmenopausal women with T2D TBS rather than BMD is associated with vertebral[53] and major osteoporotic fractures[11]. The FRAX score, being unadjusted to TBS, underestimates fracture risk in these women[54]. In our study, women with normal and reduced TBS demonstrated no differences in the unadjusted FRAX scores, although they were different in the prevalent fractures. As expected, incorporation of TBS values into the FRAX algorithm increased probability of the fractures in women with lower TBS. Therefore, TBS can help to improve the assessment of the risk of fractures in women with T2D and normal BMD. However, even after TBS

adjustment the risk of fractures may be underestimated. A recent population-based prospective study by Leslie *et al*[55] (the Manitoba BMD Registry) showed that a residual effect of diabetes on major osteoporotic fractures estimated with FRAX persists even after TBS adjustment, though the adjustment attenuated the effect of the disease. Adjustment for diabetes further improves the quality of fracture prediction.

The cross-sectional design and relatively small sample size are the limitations of our study. The recruitment of patients in one clinical center could lead to some sample bias. We could not differentiate visceral and subcutaneous adipose tissue with the applied DXA technique. As the used version of TBS iNsight software does not correct for extremes of BMI, we cannot exclude some underestimation of TBS in patients with obesity class 2 and 3[56].

At the same time, as far as we know, this is the first study estimating the risk factors for impaired bone microarchitecture assessed by TBS in postmenopausal women with T2D and normal BMD. Further studies of a larger size and prospective design are needed to establish the role of the identified factors as predictors of TBS reduction in these women. The value of TBS in the prediction of osteoporosis-related fractures in postmenopausal women with T2D and normal BMD is another challenge for future research.

CONCLUSION

In this study, we have revealed a decrease in the TBS values in 44.8% of postmenopausal women with T2D and normal BMD. These data indicate that a substantial proportion of postmenopausal women with T2D have impaired bone microarchitecture despite the normal BMD parameters. Greater height and central adiposity turned out to be the risk factors for impaired bone microarchitecture in these women. The results give further support to notion that estimation of TBS should be an essential element of DXA protocol in postmenopausal women with T2D.

ARTICLE HIGHLIGHTS

Research background

People with type 2 diabetes (T2D) have higher risk of vertebral and some non-vertebral fractures than non-diabetic individuals, regardless of normal or even increased bone mineral density (BMD). As BMD assessment may lead to underestimation of a fracture risk in T2D, additional parameters of bone health should be taken into consideration. The impaired bone microarchitecture is considered as a major contributor to fracture risk in T2D. Trabecular Bone Score (TBS) on lumbar spine dual X-ray absorptiometry (DXA) images is increasingly applied for the assessment of bone microarchitecture. Individuals with diabetes as compared to those without have significantly lower TBS.

Research motivation

At present, data on TBS in postmenopausal women with T2D and normal BMD is limited, and predictors of TBS decrease in these women need to be refined. In particular, the role of body composition and diabetes-related parameters as risk factors for deterioration of bone microarchitecture needs further research.

Research objectives

To identify clinical and body composition parameters that affect TBS in postmenopausal women with T2D and normal BMD.

Research methods

A non-interventional cross-sectional comparative study was conducted. Postmenopausal women with T2D, aged 50-75 years, with no established risk factors for secondary osteoporosis, were included. BMD, TBS and body composition parameters were assessed by DXA. In women with normal BMD, a wide range of anthropometric, general and diabetes-related clinical and laboratory parameters were evaluated as risk factors for TBS decrease using univariate and multivariate regression analysis and analysis of receiver operating characteristic (ROC) curves.

Research results

Among women with normal BMD, 44.8% showed decreased TBS values (≤ 1.31). Women with decreased TBS were taller and had a lower BMI when compared to those with normal TBS. No significant differences in HbA1c, renal function, calcium, phosphorus, alkaline phosphatase, PTH and 25(OH)D levels were found. In the models of multivariate regression analysis, TBS was positively associated with gynoid fat mass, whereas the height and androgen fat mass were associated negatively.

In the ROC-curve analysis, height ≥ 162.5 cm, body mass index < 33.85 kg/m^2, gynoid fat mass ≤ 5.41 kg and android/gynoid fat mass ratio ≥ 1.145 were identified as the risk factors for TBS reduction.

Research conclusions

The obtained results indicate that a substantial proportion of postmenopausal women with T2D and normal BMD may have impaired bone microarchitecture. Greater height and central adiposity turned out to be the risk factors for decreased TBS in these women. The results give further support to notion that estimation of TBS should be an essential element of DXA protocol in postmenopausal women with T2D.

Research perspectives

The prognostic value of TBS as a risk factor for fractures in patients with T2D and normal BMD needs further research. Prospective studies should determine the effect of changes in body weight and body composition on bone microarchitecture in these patients. The impact of hyperglycemia, glucose variability and metabolic memory, as well as various antihyperglycemic drugs, also needs to be clarified.

FOOTNOTES

Author contributions: Klimontov VV contributed to study conception and design, data analysis and interpretation; Fazullina ON and Korbut AI collected the data and performed data analysis and interpretation; Fazullina ON and Klimontov VV wrote the article; all authors approved the final version of the manuscript.

Institutional review board statement: The study protocol was approved by the Ethical Committee of the RICEL - Branch of IC&G SB RAS (protocol N. 104 from 20 December 2014).

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: Russia

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 566-580

DOI: 10.4239/wjd.v13.i7.566

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Prospective Study

Relationship between quality of life and adolescent glycolipid metabolism disorder: A cohort study

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Kuru Çolak T, Turkey; Sibiya N, South Africa; Teixeira KN, Brazil; Xiao JB, Spain

Received: December 20, 2021
Peer-review started: December 20, 2021
First decision: April 18, 2022
Revised: April 29, 2022
Accepted: June 20, 2022
Article in press: June 20, 2022
Published online: July 15, 2022

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Abstract

BACKGROUND

The prevalence of glucolipid metabolic disorders (GLMDs) in children and adolescents has a recognized association with cardiovascular diseases and type 2 diabetes mellitus in adulthood. Therefore, it is important to enhance our understanding of the risk factors for GLMD in childhood and adolescence.

AIM

To explore the relationship between quality of life (QoL) and adolescent GLMD.

METHODS

This study included 1956 samples in 2019 from a cohort study established in 2014. The QoL scale and glycolipid indexes were collected during follow-up; other covariates of perinatal factors, physical measures, and socioeconomic indicators were collected and adjusted. A generalized linear regression model and logistic regression model were used to analyse the correlation between QoL and GLMD.

RESULTS

Higher scores of QoL activity opportunity, learning ability and attitude, attitude towards doing homework, and living convenience domains correlated negatively with insulin and homeostasis model assessment insulin resistance (IR) levels. Psychosocial factors, QoL satisfaction factors, and total QoL scores had significant

protective effects on insulin and IR levels. Activity opportunity, learning ability and attitude, attitude towards doing homework domains of QoL, psychosocial factor, and total score of QoL correlated positively with high density lipoprotein. In addition, the attitude towards doing homework domain was a protective factor for dyslipidaemia, IR > 3, and increased fasting blood glucose; four factors, QoL and total QoL score correlated significantly negatively with IR > 3. In subgroup analyses of sex, more domains of QoL correlated with insulin and triglyceride levels, dyslipidaemia, and IR > 3 in females. Poor QoL was associated with an increased prevalence of GLMD, and the effect was more pronounced in males than in females. Measures to improve the QoL of adolescents are essential to reduce rates of GLMD.

CONCLUSION

Our study revealed that QoL scores mainly correlate negatively with the prevalence of GLMD in adolescents of the healthy population. The independent relationship between QoL and GLMD can be illustrated by adjusting for multiple covariates that may be associated with glycaemic index. In addition, among females, more QoL domains are associated with glycaemic index.

Key Words: Quality of life; Insulin resistance; Lipids; Metabolic abnormality

Core Tip: Persistent abnormalities of glucose and lipid metabolism in childhood have a well-established association with adulthood cardiovascular diseases. Previous conclusions about the association between quality of life (QoL) and glycolipid metabolism disorder (GLMD) were almost all based on adults with type 2 diabetes or dyslipidaemia, whereas there is limited evidence for the association between QoL and GLMD in healthy children and adolescents. This study found that a poor QoL score was associated with increased insulin, triglyceride, and IR levels, and the association was more significant in males than in females. In addition, seven domains, four factors, and total QoL score were negatively associated with abnormalities in glucose and lipid metabolism. Measures to improve the QoL of adolescents are essential to reduce the prevalence of GLMD.

Citation: Liang XH, Ren YL, Liang XY, Chen JY, Qu P, Tang X. Relationship between quality of life and adolescent glycolipid metabolism disorder: A cohort study. *World J Diabetes* 2022; 13(7): 566-580
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/566.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.566

INTRODUCTION

The increased prevalence of glucolipid metabolic disorders (GLMDs) in children and adolescents has a well-established association with cardiovascular diseases (CVDs) and type 2 diabetes (T2D) in adulthood[1]. Therefore, it is important to increase our understanding of the risk factors for GLMD during childhood and adolescence. Previous studies have illustrated that risk factors for GLMD in children include unhealthy dietary habits[2], genetic factors[3,4], poor prenatal exposure to high maternal fasting blood glucose (FBG) levels[5], and gestational diabetes. Overall, conclusions about insulin resistance (IR) and quality of life (QoL) are controversial. The results of Schlotz *et al*[6] showed that IR is associated with lower health-related QoL only in physical health domains[6]. However, a cohort study reported that participants with IR had deteriorated health-related QoL involving physical functioning, emotional role limitations, social functioning, pain, and general health perception, and a more significant correlation was found in males[7]. Several previous studies[8,9] have found that limited trials have reported health-related QoL (HRQoL) in diabetes mellitus, and diabetes affects several dimensions of QoL, such as physical, social well-being, and emotional, compared with a control group [10,11]. Additionally, the primary intervention of pravastatin plus intensive dietary advice might improve the QoL of patients with hyperlipidaemia[12,13]. Several intervention trials[10,14,15] of patients with T2D found disease special-QoL and HRQoL to be improved after treatment, accompanied by decreased FPG, triglyceride (TG), and insulin levels. A systematic review also illustrated that diabetes self-management education may improve the QoL of diabetes by decreasing glycosylated haemoglobin (HbA1c) levels[11]. Therefore, previous conclusions suggest that hyperlipidaemia and impaired fasting glycaemia may impact QoL. Moreover, a study showed that lower QoL impacts the ability to achieve a good HbA1c level[16]. Diverse QoL survey tools have been used in previous studies, with most of these assessment tools being focused on disease-specific QoL[11,17], whereas there are few

scales for measuring the global health or general health of healthy subjects[7]. QoL includes multidimensional terms, which represent satisfaction with life status and describe a subject's functioning in physical, emotional, and social domains. Little evidence about the relationship between QoL and GLMD has been reported, especially in children and adolescents, which is an important stage of growth.

To our knowledge, there are no studies exploring the correlation between QoL and GLMD in healthy children aged 10-14 years from a rural-urban cohort study. The hypothesis of this study is that QoL affect GLMD in children and adolescents. The aim of this cohort study was to explore the correlation of QoL scores with GLMD in adolescents, providing an excellent opportunity to identify independent risk factors for GLMD after adjusting for multiple variables, such as perinatal variables, socioeconomic status (SES), anthropometric measures, and other biochemical indexes.

MATERIALS AND METHODS

Subjects

Two-stage stratified cluster sampling was used to select children from urban and rural areas of Chongqing; then, two regions *per* county were randomly chosen; and finally, all children living in the selected region were informed and included if they were satisfied the inclusion criteria. In addition, a bidirectional cohort in which retrospective and prospective variables were adjusted was used to evaluate the relationship between QoL and GLMD. At baseline, children who met all the following criteria were recruited: (1) Aged 6-9 years in 2014; (2) Resided in the selected areas for more than 6 mo; (3) Did not have serious diseases (*e.g.*, nephropathy, CVD, or cancer); and (4) Consent both from the parents and children for participation. At baseline, all participants in grades 1 and 2 were recruited mainly from two elementary schools. The class head teacher delivered questionnaires to children who signed informed consent forms, and the children took the questionnaires home and completed them with their parents, after which the teacher collected the questionnaires. Two thousand one hundred and thirty-six children with venous blood samples were analysed in this study. After excluding 117 children without FBG or insulin data and 60 children without QoL information, 1959 children with complete data were analysed (Figure 1). This study was approved by the Institutional Review Board of the Children's Hospital of Chongqing Medical University, and all subjects and their parents/guardians signed informed consent forms.

Demographic variables

Demographic information and SES (parents' occupation and education level, and family income) were collected. Bachelor's and master's degrees were combined, as there were few parents with the latter. Therefore, parental education level was measured on a four-point scale [≤ 9 years (elementary and middle school), 9-12, 12-15, and > 15 years]. Perinatal variables included maternal obesity and maternal increased weight during pregnancy. Family history of obesity or CVD was investigated using a self-filled questionnaire. In addition, sleeping quality and dietary intake of vegetables, red meat, and salt were surveyed; the detailed protocol was published in a previous paper[18,19].

The questionnaire is valid and reliable, has been used in more than 20000 children, has been modified several times after each survey, and has been described in detail in our previous publications[19-21]. The questionnaire included information on demographics, perinatal status, SES, dietary intake, physical activity, and sleep quality; it was completed both by the children and their parents or guardians according to the protocol.

Physical examination

Anthropometric indexes of height, weight, and waist circumference were measured by well-trained paediatric nurses, and the detailed protocol for these measurements has been introduced in our previous papers[19,22,23].

Biochemical indexes

Venous blood (3 mL) was drawn in the morning after at least 12 h of fasting from subjects who provided informed consent[24]. FBG, total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), TGs, fasting insulin (FI), and glycosylated haemoglobin were measured within 2 h after blood draw; details are provided in other publications[19]. In 2019, FI was measured using a Siemens Centaur XP.

QoL questionnaire

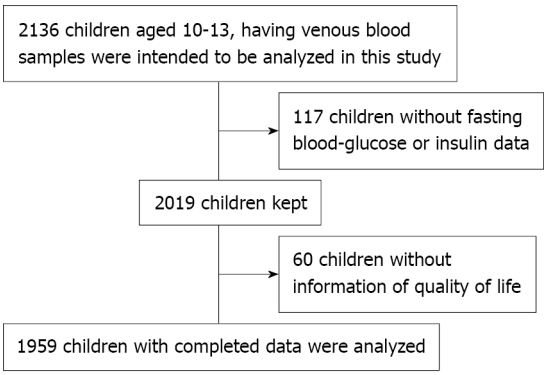
The QoL Scale for Children and Adolescents (QLSCA) with 49 items, which is suitable for children aged 7-18 years old, was used to investigate the QoL of children[25]. QLSCA includes four factors and 13 domains, which have been introduced in our previous publication[25]. A four-point scale was used with the QLSCA, with a randomized bidirectional response (positive or negative) item by item to limit the bias of the response. Individual item values were recorded in the same direction prior to analysis.

Table 1 General characteristics of glycolipid metabolism study in adolescents (mean ± SD)

Variable	Male	Female	F	*P* value
Anthropometric measures				
Age, yr	11.21 ± 0.64	11.13 ± 0.68	7.55	0.006
Height, cm	151.52 ± 8.53	152.08 ± 7.30	2.41	0.121
Weight, kg	45.31 ± 11.87	43.46 ± 10.00	13.81	< 0.001
Waist circumference, cm	68.17 ± 11.07	63.76 ± 8.52	96.30	< 0.001
FBG, mmol/L	4.49 ± 0.45	4.42 ± 0.41	14.81	< 0.001
HbA1c, %	5.37 ± 0.20	5.37 ± 0.19	0.04	0.843
Insulin, pmol/L	82.91 ± 81.09	85.30 ± 70.01	0.48	0.486
Insulin resistance index	2.42 ± 2.65	2.41 ± 2.13	0.01	0.952
Creatinine, mmol/L	52.86 ± 10.60	53.96 ± 28.20	1.26	0.261
Uric acid, μmol/L	333.51 ± 83.13	305.13 ± 66.72	65.16	< 0.001
TG, mean, mmol/L	1.04 ± 0.52	1.09 ± 0.49	4.41	0.036
HDL-C, mmol/L	1.43 ± 0.32	1.43 ± 0.30	0.00	0.994
LDL-C, mmol/L	1.84 ± 0.43	1.84 ± 0.44	0.02	0.893
Physical activity, min/day	3.52 ± 0.62	3.52 ± 0.57	0.01	0.939
Sleep score	45.08 ± 5.85	45.97± 6.38	10.36	0.001
Dietary intake, g/day				
Cereals and potatoes	183.48 ± 173.6	160.72 ± 164.5	8.86	0.003
Vegetables	207.71 ± 197.7	216.41 ± 213.8	0.88	0.349
Red meat	159.26 ± 199.9	152.11 ± 204.2	0.61	0.434
Nutritional supplements	20.05 ± 32.04	19.29 ± 32.57	0.27	0.603
Increased BMI during pregnancy, kg/m2	1.82 ± 0.75	1.82 ± 0.75	0.05	0.829
13 domains of QoL				
Self-satisfaction	50.83 ± 11.09	49.33 ± 11.48	8.60	0.003
Relationship of teacher and pupil	53.62 ± 10.20	53.81 ± 9.64	0.19	0.665
Physical feeling	50.32 ± 10.53	49.60 ± 11.00	2.16	0.142
Companionship	54.34 ± 9.88	53.14 ± 10.96	6.49	0.011
Parenthood	52.13 ± 10.83	50.73 ± 11.76	7.43	0.007
Physical activity ability	50.11 ± 10.96	50.13 ± 10.23	0.01	0.966
Learning ability and attitude	52.34 ± 9.92	51.41 ± 10.33	4.14	0.042
Self-esteem	51.18 ± 11.20	49.75 ± 10.84	8.21	0.004
Negative emotion	48.23 ± 10.79	47.14 ± 11.57	4.61	0.032
Attitude towards doing homework	51.44 ± 9.23	51.59 ± 9.00	0.13	0.716
Activity opportunity	54.99 ± 9.40	54.39 ± 9.64	1.88	0.170
Living convenience	54.41 ± 7.88	54.54 ± 7.47	0.14	0.704
Other	50.99 ± 10.01	50.60 ± 10.13	0.71	0.401
Four factors of QoL				
Psychosocial factor	64.71 ± 10.26	65.25 ± 10.17	1.36	0.244
Physical and mental health factor	36.00 ± 6.02	35.77 ± 5.90	0.73	0.393
Living environment factor	24.36 ± 4.20	23.73 ± 4.28	10.58	0.001
Quality of life satisfaction factor	25.13 ± 4.31	24.72 ± 4.42	4.38	0.037

Mother's education, yr, *n* (%)				
Approximately 9	314 (31.15)	325 (34.17)	2.35	0.308
Approximately 12	360 (35.71)	315 (33.12)		
≥ 15	334 (33.13)	311 (32.70)		
Father's education, yr, *n* (%)				
Approximately 9	277 (27.48)	250 (26.29)	6.22	0.045
Approximately 12	338 (33.53)	369 (38.80)		
≥ 15	393 (38.99)	332 (34.91)		
Income, Yuan/year, *n* (%)				
Approximately 25000	155 (15.38)	141 (14.83)	2.74	0.741
Approximately 50000	166 (16.47)	168 (17.67)		
Approximately 100000	236 (23.41)	245 (25.76)		
Approximately 150000	190 (18.85)	163 (17.14)		
Approximately 200000	117 (11.61)	106 (11.15)		
> 200000	144 (14.29)	128 (13.46)		
Region				
Urban, *n* (%)	764 (75.79)	728 (76.55)	0.16	0.694
Rural, *n* (%)	244 (24.21)	223 (23.45)		

IR: Insulin resistance; FBG: Fasting blood glucose; HbA1c: Glycosylated haemoglobin; TG: Triglyceride; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; QoL: Quality of life; BMI: Body mass index.



DOI: 10.4239/wjd.v13.i7.566

Figure 1 Flow chart of participants analyzed in this study.

Response values were summed and normalized to the age-, sex-, and urban-rural-specific norms of Chinese individuals into a score ranging from 0-100 (normalized with a mean of 50 and SD of 10), whereby higher scores represent better QoL[26]. The domain scores and factor scores between males and females are displayed in Table 1.

Diagnostic criteria

Children were diagnosed with increased blood glucose if their FBG was ≥ 5.6 mmol/L[27]. Dyslipidaemia was indicated if any one of the following criteria were met[28]: (1) TC ≥ 200 mg/dL; (2) TG ≥ 130 mg/dL; (3) LDL-C ≥ 130 mg/dL; and (4) HDL-C ≤ 40 mg/dL. Moreover, IR was defined by homeostasis model assessment (HOMA)-IR > 3.0[29], which was calculated by the formula (FI mU/L) × (FBG mmol/L)/22.5. Maternal overweight/obesity was defined as a body mass index greater than 24 kg/m^2[30]. Maternal pregnancy weight gain was defined by the guidelines of the institute of medicine [31], as gaining 12.5-18.0 kg, 11.5-16.0 kg, 7.0-11.5 kg, and 5.0-9.0 kg for underweight, normal weight, overweight, and obesity, respectively.

Table 2 Effect of dimensions of quality of life on glycolipid metabolism disorder

Variable		FI		IR		TG		HDL	
		β (95%CI)	*P* value	β (95%CI)	*P* value	β (95%CI)	*P* value	β (95%CI)	*P* value
Model 1 (domains of QoL)	Self-satisfaction	-0.399 (-0.696, -0.102)	0.009	-0.011 (-0.021, -0.001)	0.028	-0.003 (-0.005, -0.001)	0.004	0.000 (-0.001, 0.002)	0.509
	Relationship of teacher and pupil	-0.272 (-0.607, 0.063)	0.112	-0.006 (-0.017, 0.005)	0.252	-0.004 (-0.006, -0.001)	0.002	0.001 (-0.000, 0.002)	0.177
	Activity opportunity	-0.452 (-0.761, -0.143)	0.004	-0.013 (-0.023, -0.003)	0.014	-0.003 (-0.005, -0.000)	0.017	0.001 (-0.000, 0.002)	0.098
	Physical activity ability	-0.608 (-0.920, -0.295)	< 0.001	-0.014 (-0.024, -0.004)	0.008	-0.004 (-0.007, -0.002)	< 0.001	0.002 (0.001, 0.003)	0.098
	Learning ability and attitude	-0.391 (-0.721, -0.061)	0.020	-0.010 (-0.021, 0.001)	0.075	-0.002 (-0.004, 0.000)	0.109	0.002 (0.001, 0.003)	0.002
	Attitude towards doing homework	-0.684 (-1.05, -0.319)	<0.001	-0.018 (-0.030, -0.006)	0.003	-0.005 (-0.008, -0.003)	< 0.001	0.002 (0.000, 0.003)	0.033
	Living convenience	-0.469 (-0.905, -0.034)	0.035	-0.016 (-0.030, -0.001)	0.030	-0.003 (-0.005, 0.000)	0.093	0.001 (-0.001, 0.003)	0.311
Model 2 (domains of QoL)	Self-satisfaction	-0.352 (-0.641, -0.063)	0.017	-0.009 (-0.019, 0.000)	0.054	-0.003 (-0.005, -0.001)	0.006	0.001 (-0.001, 0.002)	0.330
	Relationship of teacher and pupil	-0.327 (-0.646, -0.007)	0.045	-0.008 (-0.019, 0.002)	0.127	-0.003 (-0.006, -0.001)	0.003	0.001 (-0.000, 0.002)	0.203
	Activity opportunity	-0.421 (-0.719, -0.123)	0.006	-0.012 (-0.022, -0.002)	0.018	-0.003 (-0.005, -0.001)	0.011	0.001 (0.000, 0.003)	0.027
	Physical activity ability	-0.394 (-0.696, -0.091)	0.011	-0.008 (-0.018, 0.002)	0.113	-0.003 (-0.005, -0.001)	0.005	0.001 (-0.000, 0.002)	0.192
	Learning ability and attitude	-0.442 (-0.758, -0.126)	0.006	-0.011 (-0.021, -0.000)	0.046	-0.001 (-0.004, 0.001)	0.190	0.002 (0.001, 0.004)	0.001
	Attitude towards doing homework	-0.720 (-1.07, -0.373)	< 0.001	-0.019 (-0.031, -0.008)	0.001	-0.005 (-0.008, -0.003)	< 0.001	0.002 (0.001, 0.004)	0.005
	Living convenience	-0.413 (-0.825, -0.001)	0.049	-0.014 (-0.028, -0.000)	0.043	-0.002 (-0.005, 0.001)	0.127	0.001 (-0.001, 0.003)	0.186

Model 1: Adjusted for age and sex. Model 2: Adjusted for age, sex, height, weight, vegetable intaking, red meat intaking, salt intake, sleeping quality, father's education, mother's education, household income, urban-rural areas, maternal increased weight during pregnancy, and maternal obesity. FI: Fasting insulin; IR: Insulin resistance; TG: Triglyceride; HDL: High density lipoprotein cholesterol; QoL: Quality of life.

Statistical analysis

Continuous variables, such as insulin, HOMA-IR, and TG, which did not conform to a normal distribution, were subjected to natural logarithmic transformation before analyses. The relationship between QoL and GLMD was analysed with a generalized linear model. Two models were used to adjust for covariates: Model 1 adjusted for age and sex, and Model 2 adjusted for covariates of height, weight, vegetable intake, red meat intake, salt intake, sleeping quality, father's education, mother's education, household income, urban-rural areas, maternal increased weight during pregnancy, and maternal obesity, which may reflect the independent effect of QoL on blood glucose and lipid indexes. In addition, a logistic regression model was used to analyse the relationship between QoL and GLMD with two models to adjust for covariates.

The results were analysed with SAS 9.4 software (Copyright© 2020 SAS Institute Inc. Cary, NC, United States). An α level of 0.05 was defined as a significant difference.

RESULTS

General characteristics

The general characteristics of the subjects are presented in Table 1. A total of 1956 samples were included. The mean age was 11.21 ± 0.64 years, and 51.53% (1008/1956) were males. The 13 domains, four factors, and total score of QoL, biochemical indexes, and anthropometric, perinatal, and SES variables between males and females are shown in Table 1.

Table 3 Effect of four factors of quality of life on glycolipid metabolism disorder

Variable		FI		IR		TG		HDL	
		β (95%CI)	*P* value	β (95%CI)	*P* value	β (95%CI)	*P* value	β (95%CI)	*P* value
Model 1 (four factors of QoL)	Physical and mental health factor	-0.222 (-0.547, 0.104)	0.181	-0.005 (-0.016, 0.006)	0.364	-0.003 (-0.005, -0.000)	0.017	0.001 (-0.000, 0.003)	0.064
	Psychosocial factor	-0.835 (-1.390, -0.278)	0.003	-0.021 (-0.039, -0.002)	0.026	-0.006 (-0.010, -0.002)	0.002	0.002 (-0.000, 0.005)	0.052
	Living environment factor	-0.821 (-1.610, -0.036)	0.040	-0.014 (-0.039, 0.012)	0.298	-0.007 (-0.012, -0.002)	0.012	0.004 (0.001, 0.008)	0.008
	Quality of life satisfaction factor	-0.848 (-1.610, -0.082)	0.030	-0.023 (-0.048, 0.002)	0.074	-0.006 (-0.011, -0.001)	0.031	0.001 (-0.002, 0.004)	0.507
	Total score of QoL	-0.440 (-0.710, -0.169)	0.002	-0.012 (-0.020, -0.003)	0.011	-0.004 (-0.006, -0.002)	< 0.001	0.001 (0.000, 0.002)	0.040
Model 2 (four factors of QoL)	Physical and mental health factor	-0.272 (-0.588, 0.044)	0.092	-0.006 (-0.017, 0.004)	0.229	-0.002 (-0.005, -0.000)	0.039	0.001 (-0.000, 0.002)	0.122
	Psychosocial factor	-0.906 (-1.450, -0.367)	0.001	-0.023 (-0.041, -0.005)	0.011	-0.007 (-0.010, -0.003)	0.001	0.003 (0.001, 0.005)	0.008
	Living environment factor	-0.916 (-1.690, -0.143)	0.020	-0.018 (-0.044, 0.008)	0.172	-0.005 (-0.011, 0.000)	0.067	0.003 (-0.001, 0.006)	0.126
	Quality of life satisfaction factor	-0.965 (-1.710, -0.217)	0.012	-0.027 (-0.052, -0.002)	0.035	-0.006 (-0.011, -0.001)	0.027	0.002 (-0.001, 0.005)	0.239
	Total score of QoL	-0.441 (-0.705, -0.177)	0.001	-0.011 (-0.020, -0.003)	0.010	-0.004 (-0.006, -0.002)	0.001	0.001 (0.000, 0.002)	0.021

Model 1: Adjusted for age and sex. Model 2: Adjusted for age, sex, height, weight, vegetable intaking, red meat intaking, salt intake, sleeping quality, father's education, mother's education, household income, urban-rural areas, maternal increased weight during pregnancy, and maternal obesity. FI: Fasting insulin; IR: Insulin resistance; TG: Triglyceride; HDL: High density lipoprotein cholesterol; QoL: Quality of life.

Association between QoL and glycolipid indexes

Tables 2 and 3 display the effect of QoL on glycolipid metabolism in adolescents. Adolescents with higher domain scores of living convenience had lower FBG than their counterparts ($P < 0.05$). TG and HDL-C were higher in adolescents who had a negative attitude towards doing homework ($P < 0.05$), and the impact of living convenience and attitude towards doing homework on glycolipid indexes (TG and HDL-C) was also significant after adjusting for multiple factors. However, the impact of QoL factors on LDL-C and TC was not significant ($P > 0.05$) (Supplementary Tables 1 and 2). Levels of insulin and IR were lower in adolescents with a higher factor score of psychosocial, living environment, and QoL satisfaction than their counterparts ($P < 0.05$). Moreover, adolescents with higher psychosocial factor scores and total QoL scores had decreased TGs and increased HDL-C compared with their counterparts after adjusting for covariates ($P < 0.05$).

The effect of QoL on glycolipid metabolism indexes by sex is shown in Supplementary Tables. The results in Supplementary Tables 3 and 4 illustrate the relationship between QoL and indexes (FI, IR, TG, and HDL). Scores of attitude towards doing homework and living convenience were negative for FI, IR, and TG ($P < 0.05$ or $P < 0.001$), and the association of attitude towards doing homework with IR/TG was also significant after adjusting for covariates in Model 2 ($P < 0.05$). Moreover, the relationship between total QoL score and FI/TG was negative ($P < 0.05$). Higher scores of activity opportunity, physical activity ability, learning ability, and attitude and lower levels of FI, TG, and HDL were found for females ($P < 0.05$); in Model 2, the score of attitude towards doing homework correlated negatively with IR level ($P = 0.018$).

The results showed that the total score of QoL was a negative factor for FI [β (95%CI): -0.441 (-0.705, -0.177)], IR [β (95%CI): -0.011 (-0.020, -0.003)], and TG [β (95%CI): -0.004 (-0.006, -0.002)] but a positive factor for HDL [β (95%CI): 0.001 (0.000, 0.002)].

In addition, the association between the four factors of QoL and the prevalence of glycolipid metabolism indexes by sex is shown in Supplementary Tables 5 and 6. The relationship between the living convenience score and FBG was negative ($P < 0.05$). However, significant relationships for females were only found in Model 1.

Association between QoL and GLMD prevalence

The results in Tables 4 and 5 indicated the relationship between QoL and GLMD in adolescents. The attitude towards doing homework domain score was a protective factor for dyslipidaemia [OR (95%CI): 0.984 (0.972, 0.995); $P = 0.004$], and the relationship was significant even after adjusting for covariates

Table 4 Logistic regression analysis of dimensions of quality of life and glycolipid metabolism disorder

Variable		Dyslipidemia		IR > 3		Increased FBG	
		OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Total							
Model 1 (domains of QoL)	Self-satisfaction	0.994 (0.984, 1.003)	0.177	0.987 (0.978, 0.997)	0.009	1.009 (0.991, 1.027)	0.344
	Relationship of teacher and pupil	0.991 (0.981, 1.002)	0.095	0.987 (0.977, 0.998)	0.025	1.012 (0.991, 1.032)	0.264
	Activity opportunity	0.989 (0.980, 0.999)	0.031	0.988 (0.978, 0.998)	0.023	1.006 (0.988, 1.025)	0.527
	Physical activity ability	0.988 (0.978, 0.998)	0.020	0.981 (0.971, 0.992)	0.001	1.013 (0.994, 1.031)	0.184
	Learning ability and attitude	0.990 (0.979, 1.000)	0.057	0.989 (0.978, 1.000)	0.047	1.003 (0.984, 1.023)	0.736
	Attitude towards doing homework	0.984 (0.972, 0.995)	0.004	0.986 (0.974, 0.998)	0.026	0.978 (0.959, 0.997)	0.022
	Living convenience	0.997(0.984,1.011)	0.712	0.990(0.976,1.004)	0.158	0.993(0.970,1.018)	0.587
Model 2 (domains of QoL)	Self-satisfaction	0.993 (0.984, 1.003)	0.183	0.986 (0.975, 0.996)	0.007	1.008 (0.989, 1.026)	0.408
	Relationship of teacher and pupil	0.992 (0.981, 1.003)	0.154	0.984 (0.972, 0.996)	0.008	1.007 (0.987, 1.029)	0.480
	Activity opportunity	0.988 (0.978, 0.998)	0.017	0.986 (0.975, 0.997)	0.016	1.007 (0.988, 1.026)	0.497
	Physical activity ability	0.994 (0.984, 1.005)	0.268	0.985 (0.973, 0.997)	0.012	1.013 (0.994, 1.033)	0.184
	Learning ability and attitude	0.990 (0.979, 1.001)	0.072	0.986 (0.974, 0.998)	0.018	1.003 (0.983, 1.023)	0.770
	Attitude towards doing homework	0.982 (0.970, 0.994)	0.003	0.983 (0.971, 0.996)	0.012	0.978 (0.958, 0.997)	0.024
	Living convenience	0.997 (0.983, 1.011)	0.685	0.990 (0.975, 1.005)	0.195	0.996 (0.972, 1.021)	0.765
Male							
Model 1 (domains of QoL)	Self-satisfaction	0.996 (0.983, 1.009)	0.538	0.990 (0.977, 1.004)	0.173	1.001 (0.980, 1.023)	0.927
	Relationship of teacher and pupil	0.998 (0.983, 1.012)	0.759	0.994 (0.978, 1.009)	0.414	1.003 (0.979, 1.027)	0.836
	Activity opportunity	0.995 (0.981, 1.009)	0.478	0.993 (0.978, 1.008)	0.370	0.999 (0.977, 1.022)	0.942
	Physical activity ability	0.993 (0.980, 1.007)	0.341	0.984 (0.970, 0.999)	0.037	1.006 (0.984, 1.028)	0.623
	Learning ability and attitude	0.996 (0.981, 1.011)	0.597	0.999 (0.983, 1.016)	0.915	0.992 (0.969, 1.016)	0.503
	Attitude towards doing homework	0.983 (0.968, 0.998)	0.031	0.996 (0.979, 1.013)	0.636	0.980 (0.957, 1.004)	0.102
	Living convenience	1.008 (0.988, 1.027)	0.444	0.988 (0.968, 1.008)	0.229	0.982 (0.955, 1.010)	0.211
Model 2 (domains of QoL)	Self-satisfaction	0.996 (0.982, 1.010)	0.570	0.991 (0.976, 1.006)	0.241	1.003 (0.981, 1.026)	0.766
	Relationship of teacher and pupil	0.999 (0.984, 1.014)	0.888	0.991 (0.975, 1.008)	0.313	1.000 (0.976, 1.025)	0.982
	Activity opportunity	0.993 (0.978, 1.008)	0.365	0.992 (0.976, 1.008)	0.332	1.004 (0.981, 1.027)	0.762
	Physical activity ability	1.002 (0.988, 1.017)	0.745	0.991 (0.975, 1.007)	0.282	1.009 (0.986, 1.033)	0.438
	Learning ability and attitude	0.996 (0.981, 1.012)	0.645	0.998 (0.980, 1.015)	0.793	0.994 (0.970, 1.018)	0.604
	Attitude towards doing homework	0.980 (0.964, 0.996)	0.017	0.996 (0.977, 1.014)	0.650	0.983 (0.959, 1.008)	0.180
	Living convenience	1.013 (0.992, 1.034)	0.222	0.994 (0.973, 1.016)	0.603	0.987 (0.958, 1.016)	0.363
Female							

Model 1 (domains of QoL)	Self-satisfaction	0.991 (0.978, 1.004)	0.195	0.985 (0.972, 0.998)	0.022	1.024 (0.992, 1.056)	0.146
	Relationship of teacher and pupil	0.983 (0.968, 0.999)	0.035	0.980 (0.965, 0.996)	0.012	1.029 (0.991, 1.068)	0.139
	Activity opportunity	0.984 (0.971, 0.998)	0.021	0.984 (0.970, 0.998)	0.021	1.017 (0.986, 1.050)	0.292
	Physical activity ability	0.982 (0.967, 0.997)	0.016	0.977 (0.962, 0.993)	0.004	1.025 (0.993, 1.058)	0.133
	Learning ability and attitude	0.984 (0.969, 0.999)	0.032	0.980 (0.965, 0.995)	0.009	1.023 (0.991, 1.057)	0.164
	Attitude towards doing homework	0.984 (0.968, 1.001)	0.062	0.977 (0.960, 0.994)	0.007	0.972 (0.942, 1.004)	0.084
	Living convenience	0.986 (0.967, 1.006)	0.174	0.991 (0.971, 1.012)	0.387	1.018 (0.972, 1.067)	0.448
Model 2 (domains of QoL)	Self-satisfaction	0.991 (0.978, 1.005)	0.230	0.981 (0.966, 0.996)	0.011	1.014 (0.981, 1.049)	0.404
	Relationship of teacher and pupil	0.985 (0.969, 1.001)	0.060	0.975 (0.958, 0.992)	0.005	1.017 (0.977, 1.058)	0.420
	Activity opportunity	0.984 (0.970, 0.998)	0.026	0.982 (0.967, 0.997)	0.022	1.009 (0.977, 1.043)	0.573
	Physical activity ability	0.986 (0.971, 1.002)	0.097	0.976 (0.959, 0.994)	0.007	1.019 (0.983, 1.056)	0.307
	Learning ability and attitude	0.984 (0.969, 1.000)	0.046	0.974 (0.958, 0.991)	0.003	1.016 (0.982, 1.051)	0.362
	Attitude towards doing homework	0.983 (0.966, 1.000)	0.052	0.972 (0.954, 0.991)	0.003	0.965 (0.934, 0.997)	0.031
	Living convenience	0.983 (0.963, 1.004)	0.106	0.984 (0.963, 1.007)	0.167	1.013 (0.964, 1.064)	0.613

Model 1: Adjusted for age and sex. Model 2: Adjusted for age, sex, height, weight, vegetable intaking, red meat intaking, salt intake, sleeping quality, father's education, mother's education, household income, urban-rural areas, maternal increased weight during pregnancy, and maternal obesity. IR: Insulin resistance; FBG: Fasting blood glucose; QoL: Quality of life.

[OR (95%CI): 0.982 (0.970, 0.994); P = 0.003]. This relationship was also statistically significant in males [OR (95%CI): 0.983 (0.968, 0.998); P = 0.031]. However, among females, there were other factors of significance, such as relationship of teacher and pupil [OR (95%CI): 0.985 (0.969, 1.001); P = 0.060], activity opportunity [OR (95%CI): 0.984 (0.970, 0.998); P = 0.026], and learning ability and attitude [OR (95%CI): 0.984 (0.969, 1.000); P = 0.046]. After adjusting for covariates in Model 1 and Model 2, self-satisfaction, the relationship of teacher and pupil, activity opportunity, physical activity ability, learning ability and attitude, and attitude towards doing homework were protective factors for IR > 3 in all participants and in females, but only the physical activity ability domain score was significant in males [OR (95%CI): 0.984 (0.970, 0.999); P = 0.037] in Model 1. Attitude towards the homework domain was a protective factor for FBG in Model 2 for all subjects and females (P < 0.05) (Table 4).

The relationship between the four factors of QoL and the prevalence of GLMD was not significant in males (P > 0.05). Psychosocial factor [OR (95%CI): 0.976 (0.959, 0.994); P = 0.008] [OR (95%CI): 0.980 (0.962, 0.998); P = 0.034] and total score of QoL [OR (95%CI): 0.990 (0.982, 0.999); P = 0.025] [OR (95%CI): 0.988 (0.979, 0.997); P = 0.007] were protective factors for dyslipidaemia and IR > 3, respectively, with statistical significance in the total cohort in Model 1. In addition, higher score of psychosocial factor [OR (95%CI): 0.971 (0.946, 0.996); P = 0.024], living environment factor [OR (95%CI): 0.952 (0.919, 0.987); P = 0.008], and total score of QoL [OR (95%CI): 0.985 (0.973, 0.997); P = 0.014] in females was related to a lower prevalence of dyslipidaemia in Model 2. In Model 2, adjusted for covariates, all factors of QoL were protective factors for IR > 3 (P < 0.05) (Table 5).

DISCUSSION

The association between QoL and the prevalence of GLMD was illustrated using a large-sample-size childhood health cohort study. By adjusting for multiple covariates that may correlate with glycolipid indexes, the independent relationship between QoL and GLMD was demonstrated. In addition, more domains of QoL correlated with glycolipid indexes in females.

Our study revealed that QoL scores mainly correlate negatively with the prevalence of GLMD in adolescents. Research on the relationship between QoL and GLMD in the healthy population is limited. According to a previous cross-sectional study that included 74 diabetic adolescents[32], no significant relationship between QoL and HbA1c levels was observed. However, a cross-sectional study found that QoL scores correlated with an increase in the components of MS, with the physical health domain of

Table 5 Logistic regression analysis of four factors of quality of life and glycolipid metabolism disorder

Variables		Dyslipidemia		IR		Increased FBG	
		OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Total							
Model 1 (four factors of QoL)	Physical and mental health factor	0.992 (0.982, 1.002)	0.120	0.991 (0.980, 1.002)	0.113	1.020 (1.000, 1.041)	0.049
	Psychosocial factor	0.976 (0.959, 0.994)	0.008	0.980 (0.962, 0.998)	0.034	0.995 (0.964, 1.027)	0.767
	Living environment factor	0.977 (0.952, 1.001)	0.065	0.979 (0.954, 1.006)	0.121	1.077 (1.026, 1.130)	0.003
	Quality of life satisfaction factor	0.992 (0.968, 1.017)	0.531	0.972 (0.948, 0.997)	0.028	1.033 (0.986, 1.082)	0.168
	Total score of QoL	0.990 (0.982, 0.999)	0.025	0.988 (0.979, 0.997)	0.007	1.009 (0.993, 1.025)	0.267
Model 2 (four factors of QoL)	Physical and mental health factor	0.994 (0.983, 1.005)	0.296	0.988 (0.976, 1.000)	0.047	1.015 (0.994, 1.036)	0.154
	Psychosocial factor	0.973 (0.953, 0.993)	0.010	0.973 (0.953, 0.993)	0.010	0.992 (0.960, 1.025)	0.620
	Living environment factor	0.988 (0.962, 1.015)	0.387	0.971 (0.942, 1.000)	0.053	1.060 (1.008, 1.115)	0.024
	Quality of life satisfaction factor	0.992 (0.966, 1.017)	0.520	0.961 (0.934, 0.988)	0.005	1.024 (0.977, 1.074)	0.320
	Total score of QoL	0.991 (0.982, 1.000)	0.043	0.985 (0.976, 0.995)	0.004	1.009 (0.992, 1.026)	0.317
Male							
Model 1 (four factors of QoL)	Physical and mental health factor	0.999 (0.985, 1.014)	0.899	0.999 (0.983, 1.015)	0.913	1.016 (0.992, 1.042)	0.196
	Psychosocial factor	0.981 (0.957, 1.005)	0.124	0.998 (0.972, 1.025)	0.889	0.982 (0.945, 1.021)	0.355
	Living environment factor	1.001 (0.966, 1.038)	0.935	0.987 (0.950, 1.026)	0.514	1.060 (0.998, 1.125)	0.057
	Quality of life satisfaction factor	1.002 (0.968, 1.037)	0.916	0.986 (0.950, 1.023)	0.459	1.018 (0.962, 1.077)	0.537
	Total score of QoL	0.995 (0.983, 1.008)	0.470	0.995 (0.982, 1.009)	0.482	1.003 (0.983, 1.023)	0.799
Model 2 (four factors of QoL)	Physical and mental health factor	1.002 (0.986, 1.017)	0.824	0.999 (0.982, 1.016)	0.895	1.013 (0.988, 1.039)	0.301
	Psychosocial factor	0.994 (0.965, 1.024)	0.686	0.994 (0.965, 1.024)	0.686	0.983 (0.945, 1.024)	0.414
	Living environment factor	1.021 (0.982, 1.062)	0.299	0.989 (0.947, 1.033)	0.633	1.047 (0.983, 1.114)	0.154
	Quality of life satisfaction factor	1.001 (0.964, 1.039)	0.969	0.982 (0.943, 1.022)	0.374	1.016 (0.959, 1.077)	0.580
	Total score of QoL	0.997 (0.984, 1.010)	0.645	0.996 (0.982, 1.011)	0.601	1.005 (0.984, 1.026)	0.631
Female							
Model 1 (four factors of QoL)	Physical and mental health factor	0.984 (0.970, 0.999)	0.037	0.983 (0.968, 0.998)	0.026	1.025 (0.990, 1.061)	0.160
	Psychosocial factor	0.971 (0.946, 0.996)	0.024	0.962 (0.936, 0.988)	0.004	1.020 (0.965, 1.079)	0.477
	Living environment factor	0.952 (0.919, 0.987)	0.008	0.970 (0.935, 1.006)	0.105	1.102 (1.016, 1.195)	0.019
	Quality of life satisfaction factor	0.983 (0.950, 1.017)	0.329	0.958 (0.925, 0.992)	0.016	1.056 (0.976, 1.143)	0.173

	Total score of QoL	0.985 (0.973, 0.997)	0.014	0.980 (0.968, 0.993)	0.002	1.020 (0.992, 1.049)	0.156
Model 2 (four factors of QoL)	Physical and mental health factor	0.987 (0.971, 1.003)	0.103	0.976 (0.960, 0.993)	0.007	1.011 (0.974, 1.049)	0.565
	Psychosocial factor	0.956 (0.929, 0.984)	0.003	0.956 (0.929, 0.984)	0.003	1.006 (0.950, 1.065)	0.846
	Living environment factor	0.962 (0.925, 1.000)	0.048	0.951 (0.912, 0.992)	0.019	1.072 (0.982, 1.171)	0.120
	Quality of life satisfaction factor	0.984 (0.948, 1.021)	0.393	0.941 (0.905, 0.978)	0.002	1.025 (0.942, 1.115)	0.568
	Total score of QoL	0.985 (0.972, 0.998)	0.024	0.975 (0.961, 0.989)	0.001	1.012 (0.982, 1.043)	0.445

Model 1: Adjusted for age and sex. >Model 2: Adjusted for age, sex, height, weight, vegetable intaking, red meat intaking, salt intake, sleeping quality, father's education, mother's education, household income, urban-rural areas, maternal increased weight during pregnancy, and maternal obesity. IR: Insulin resistance; FBG: Fasting blood glucose; QoL: Quality of life.

QoL having the most significant association[33]. In our study, six domains, four factors, and the total QoL score were correlated significantly negatively with glycolipid indexes, and the effect was independent of obesity. To our knowledge, this is the first cohort study with a large sample size to explore the relationship of QoL with GLMD in adolescents.

The association between QoL and GLMD may be impacted by sex. Scores on several domains of QoL are reportedly lower in females than in males[34-37]. Longitudinal studies have shown a significant relationship between weight and QoL only in females[38]. However, one study found that females and males have similar psychological characteristics[39]. Overall, numerous studies have detected significant sex differences in awareness and mental health[40,41]. For instance, in terms of personality, males score higher than females in self-acceptance and autonomy, whereas females score higher than males in personal growth and positive relationships with others[42]. Our study adds more evidence about the sex difference in the association between QoL and GLMD; overall, more domains of QoL correlated with GLMD in females.

Several mechanisms may explain why QoL may impact GLMD. Physical and psychological health and social well-being are encompassed in HRQoL[43]. Previous study results show that an increase in total HbA1c is related to a decrease in QoL[44]. In addition, research has found that better QoL is associated with better healthy dietary patterns and behaviours in children and adolescents[45]. Irrational diets may induce FBG increases. For example, a high-fat diet induces IR, triggering accumulation of diacylglycerol and ceramide levels in the liver and inhibiting the insulin signalling pathway [46]. Some studies have suggested that physical activity and mental health are positively associated with QoL[44]; it is well known that exercise enhances insulin signalling independent of PI3K and that glucose transport and GLUT4 translocation are enhanced as skeletal muscle contraction is stimulated by insulin [47]. Similarly, better education may lead to greater confidence, a sense of security, and building better relationships with others, contributing to mental health[48]. The mechanisms through which IR influences emotional regulation are being revealed by animal and human studies, and the brain requires glucose as an essential energy source[49,50].

In conclusion, GLMD prevalence and high glycolipid levels are elevated in adolescents with low QoL scores. To our knowledge, this is the first study to explore the relationship of QoL with glycolipid indexes from a large-sample-size cohort study of adolescents, and the correlation was significant after adjusting for multiple covariates. Our study emphasizes the importance of improving QoL in children and adolescents and provides scientific evidence for educational institutions to improve the educational model to enhance the QoL of school-age children. However, our study illustrates the relationship between QoL and glycolipid indexes from a nearly cross-sectional perspective, and a further well-designed large-sample-size cohort study or randomized controlled trial study should be conducted to examine the causal relationships.

CONCLUSION

Our study reveals that QoL scores mainly correlate negatively with the prevalence of GLMD in adolescents of the healthy population. The independent relationship between QoL and GLMD can be illustrated by adjusting for multiple covariates that may be associated with glycaemic index. In addition, more QoL domains are associated with glycaemic index in females.

ARTICLE HIGHLIGHTS

Research background

The prevalence of glucolipid metabolic disorders (GLMDs) in children and adolescents has a recognized association with cardiovascular diseases and type 2 diabetes mellitus in adulthood. Therefore, it is important to increase our understanding of the risk factors for GLMD in childhood and adolescence.

Research motivation

Quality of life (QoL) includes multidimensional terms, which represent satisfaction with life status and describe a subject's functioning in physical, emotional, and social domains. Little evidence about the relationship between QoL and GLMD has been reported, especially in children and adolescents, which is an important stage of growth.

Research objectives

The aim of this cohort study was to explore the correlation of QoL scores and personality traits with GLMD in adolescents, providing an excellent opportunity to identify independent risk factors for GLMD after adjusting for multiple variables, such as perinatal variables, socioeconomic status, anthropometric measures, and other biochemical indexes.

Research methods

Two-stage stratified cluster sampling was used to select children from urban and rural areas of Chongqing; two regions *per* county were randomly chosen; and finally, all children living in the selected region were informed and included if they met the inclusion criteria.

Research results

Our study revealed that QoL scores mainly correlate negatively with the prevalence of GLMD in adolescents.

Research conclusions

The prevalence of GLMD and high glycolipid levels are increased in adolescents with features of low QoL scores. Our study adds more evidence about sex difference in the association between QoL and GLMD, and more domains of QoL correlate with GLMD in females.

Research perspectives

Our study illustrates the relationship between QoL and glycolipid indexes from a nearly cross-sectional perspective, and a further well-designed cohort study with a large sample size or randomized controlled trial should be conducted to explore the causal relationships.

FOOTNOTES

Author contributions: Liang XH conceived of and designed the study; Qu P and Chen JY participated in the acquisition of the data; Liang XH analysed the data; Liang XH, Ren YL, and Liang XY drafted and revised the manuscript; all authors critically reviewed and approved the final paper.

Supported by Intelligent Medicine Research Project of Chongqing Medical University, No. ZHYX202109; The Major Health Project of Chongqing Science and Technology Bureau, No. CSTC2021jscx-gksb-N0001; Research and Innovation Team of Chongqing Medical University, No. W0088; Joint Medical Research Project of Chongqing Municipal Health Commission and Chongqing Science and Technology Bureau, No. 2020MSXM062; National Key Research and Development Project of the Ministry of Science and Technology of the People's Republic of China, No. 2017YFC0211705; and Young Scientists Fund Program of the National Natural Science Foundation of China, No. 81502826.

Institutional review board statement: The study was reviewed and approved by the Chongqing Jiulongpo District Center for Disease Control and Prevention Institutional Review Board, No. 2013-006.

Clinical trial registration statement: This study is registered at ChiCTR. The registration identification number is ChiCTR2100048861.

Informed consent statement: Informed consent was obtained from all patients for inclusion in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Anonymised participant data will be shared after approval by the corresponding author, following a reasonable submitted request. Data are available from Xiao-Hua Liang (Clinical Epidemiology and Biostatistics Department, Children's Hospital of Chongqing Medical University, No.136 2nd Zhongshan Road, Yuzhong District, Chongqing, China, 400016; Email: xiaohualiang@hospital.cqmu.edu.cn or liangxiaohua666@sina.com).

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

Country/Territory of origin: China

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S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 July 15; 13(7): 581-583

DOI: 10.4239/wjd.v13.i7.581

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

More studies are necessary to establish the effectiveness of Jinhuang powder in the treatment of diabetic foot

Ya-Wen Ye, Zi-Yun Yan, Lian-Ping He, Cui-Ping Li

Specialty type: Peripheral vascular disease

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Corvino A, Italy; Primadhi RA, Indonesia; Złoch M
A-Editor: Lin FY, China

Received: February 28, 2022
Peer-review started: February 28, 2022
First decision: April 17, 2022
Revised: April 18, 2022
Accepted: June 14, 2022
Article in press: June 14, 2022
Published online: July 15, 2022

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Abstract

Diabetes mellitus is a common global public health problem that can cause serious illness and premature death. Diabetic foot ulcer, one of the complications of diabetes, is a major cause of morbidity and mortality and is associated with many other devastating complications. Previous study found that a group of traditional Chinese medicine (TCM) can be used for treating diabetic foot ulcers. More and more attention is being paid to the use of Chinese medicine to heal diabetic feet. Under the guidance of relevant theories of traditional Chinese medicine, more studies are needed to reveal the key active components and related signal pathways of TCM in the treatment of diabetic foot ulcer. One clinical study explored the treatment of diabetic foot with infection combined moist exposed burn ointment with Jinhuang powder. However, large-scale multi-center, double blind, randomized, placebo-controlled clinical trials and animal studies are necessary to establish the effectiveness of Jinhuang powder in the treatment of diabetic foot.

Key Words: Diabetic foot; Jinhuang powder; Traditional Chinese medicine

Core Tip: In recent years, most diabetic foot patients in China are adopting traditional Chinese medicine and western medicine. The short duration of clinical follow-up was not sufficient to explain the efficacy of the treatment, and the safety of the treatment was not mentioned. Large multicenter, double-blind, randomized, placebo controlled clinical trials and animal studies are necessary to determine Jinhuang powder as supplement combined with other Chinese medicine or western medicine as an effective and safe therapy for diabetic foot.

Citation: Ye YW, Yan ZY, He LP, Li CP. More studies are necessary to establish the effectiveness of Jinhuang powder in the treatment of diabetic foot. *World J Diabetes* 2022; 13(7): 581-583
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/581.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.581

TO THE EDITOR

This is a comment on "This is a comment on "Clinical Study of moist exposed burn ointment (MEBO) Combined with Jinhuang powder for Diabetic Foot with Infection"[1]. We were pleased that read the research article by Hong-Bo Zhan, *et al*[1]. Their work highlights that the use of Jinhuang powder as supplement combined with MEBO as an effective and safe therapy for diabetic foot. This study provides important clues to the treatment of foot infection, ulcer.

Diabetic foot ulcers are one of the most challenging complications of diabetes[2-4]. Previous study found that a group of traditional Chinese medicine (TCM) (*e.g.*, herbal medicine foot bath decoction[5], TCM injections[6,7], Chinese herbal medicine ulcer oil[8], moxibustion[9], Astragali Radix and Rehmanniae Radix Mixture[10], the peptide compounds of Wuguchong[11], Astragali Radix and Rehmanniae Radix[12,13]) played an important role in the treatment of the disease. Some study also found that interventional radiology plays a crucial role in the treatment of diabetic foot disease[14]. However, the study only focuses on Jinhuang powder has improved the efficacy and safety of MEBO in the treatment of diabetic foot. Thus, some questions still need further be discussed.

In recent years, most diabetic foot patients in China are adopting TCM and western medicine[5,15]. A series of systematic review articles showed that TCM can increase the clinical effective rate of conventional therapies by 27%[6], regulate the signaling pathways to promote diabetic wound healing[16]. An experiment on albino Wistar rats found that Astragali Radix and Rehmanniae Radix in the ratio of 2:1 significantly enhance the circulating CD34+/VEGFR2+/CD45-EPCs levels in diabetic foot ulcer[17]. Another study confirmed the effect of the peptide compounds of Wuguchong in treating diabetic ulcers to a certain extent[11]. However, therapeutic effect criterion is observing wound surface and assessing degree of pain. The research evaluation index was single and lacked objective evaluation.

Another problem of this study was research design. It was only a single-center trial, no double blindness, no placebo group. The short duration of clinical follow-up was not sufficient to explain the efficacy of the treatment, and the safety of the treatment was not mentioned. Large multicenter, double-blind, randomized, placebo controlled clinical trials and animal studies are necessary to determine Jinhuang powder as supplement combined with other Chinese medicine or western medicine as an effective and safe therapy for diabetic foot.

Increasing attention is being given to the use of Chinese medicine for healing diabetic foot. Under the guidance of relevant theories of TCM, more studies are needed to reveal the key active components and related signal pathways of TCM in the treatment of diabetic foot ulcer, so as to promote the further research and clinical application of TCM[16].

Overall, MEBO combined with Jinhuang powder is more effective than MEBO alone in treating diabetic foot. However, large-scale multi-center, double blind, randomized, placebo-controlled clinical trials and animal studies are also necessary to establish the effectiveness of Jinhuang powder in the treatment of diabetic foot.

FOOTNOTES

Author contributions: Li CP and He LP conceived of the presented idea and provided critical feedback to the final manuscript; Li CP, Ye YW and Yan ZY wrote and revised the manuscript; Li CP and He LP approved the main conceptual ideas and proof outline; all authors provided final edits and approved the manuscript.

Supported by the research work was supported by the Taizhou philosophy and social science planning project, No. 19GHZ05; and the Taizhou Science and technology planning project, No. 1902ky85.

Conflict-of-interest statement: The authors have no conflict of interest to disclose.

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

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World J Diabetes 2022 July 15; 13(7): 584-586

DOI: 10.4239/wjd.v13.i7.584

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Epidemiology for public health practice: The application of spatial epidemiology

Longjian Liu, Garvita Nagar, Ousmane Diarra, Stephanie Shosanya, Geeta Sharma, David Afesumeh, Akshatha Krishna

Specialty type: Health care sciences and services

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A, A
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Aslam MS, Malaysia; Fakhradiyev I, Kazakhstan; Harsini PA, Iran; Kadriyan H, Indonesia; Mohammadi A; Welter J, Switzerland

Received: February 12, 2022
Peer-review started: February 12, 2022
First decision: April 17, 2022
Revised: May 5, 2022
Accepted: June 22, 2022
Article in press: June 22, 2022
Published online: July 15, 2022

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Abstract

Spatial epidemiology is the description and analysis of geographic patterns and variations in disease risk factors, morbidity and mortality with respect to their distributions associated with demographic, socioeconomic, environmental, health behavior, and genetic risk factors, and time-varying changes. In the Letter to Editor, we had a brief description of the practice for the mortality and the space-time patterns of John Snow's map of cholera epidemic in London, United Kingdom in 1854. This map is one of the earliest public heath practices of developing and applying spatial epidemiology. In the early history, spatial epidemiology was predominantly applied in infectious disease and risk factor studies. However, since the recent decades, noncommunicable diseases have become the leading cause of death in both developing and developed countries, spatial epidemiology has been used in the study of noncommunicable disease. In the Letter, we addressed two examples that applied spatial epidemiology to cluster and identify stroke belt and diabetes belt across the states and counties in the United States. Similar to any other epidemiological study design and analysis approaches, spatial epidemiology has its limitations. We should keep in mind when applying spatial epidemiology in research and in public health practice.

Key Words: Diabetes mellitus; Spatial epidemiology; Diabetes belt; Public health practice

Core Tip: This is a Letter to the Editor on the article published in *World Journal of Diabetes* 2021; 12: 1042, entitled: Spatial epidemiology of diabetes: Methods and Insights. Spatial epidemiology is a new sub-field of epidemiology. We read with great interest this paper, and would like to further address the application of spatial epidemiology for public health practice.

Citation: Liu L, Nagar G, Diarra O, Shosanya S, Sharma G, Afesumeh D, Krishna A. Epidemiology for public health practice: The application of spatial epidemiology. *World J Diabetes* 2022; 13(7): 584-586
URL: https://www.wjgnet.com/1948-9358/full/v13/i7/584.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i7.584

TO THE EDITOR

Dear Editor, we read with great interest the recently published review paper, entitled "Spatial epidemiology of diabetes: Methods and Insights" by Cuadros *et al*[1], in *World Journal of Diabetes* 2021. The investigators reviewed spatial methods used to understand the spatial structure of the disease and identify the potential geographical drivers of the spatial distribution of diabetes mellitus. Their report serves as a good review on the concepts and methods of spatial epidemiology. In this letter we aimed to briefly address few examples of the historical public health practice in the United Kingdom and the application of spatial epidemiology in the recent decades in the United States, and to address the potential limitations when applying this technique in research and in public health practice.

The method used in "*analysis of geographic variation in disease*" could be tracked back to more than 150 years ago, for example, the renowned study of cholera epidemic in London, United Kingdom In 1854. John Snow, a physician, used mapping approach to trace the source of the Broad Street cholera outbreak (or Golden Square outbreak) in central London[1]. In the United States, an example is that a "stroke belt" or "stroke alley" was identified in early 1980s using spatial analysis approach and to define a 11-state region, where the states had age-adjusted stroke mortality rates more than 10% above the national average[2,3]. In 2011, a study by Barker *et al*[4] identified a geographically coherent region of the United States, where the prevalence of diagnosed diabetes mellitus is especially high. This area is also known as the "diabetes belt". The "diabetes belt" consisted of 644 counties in 15 mostly southern states. A further analysis indicated that the prevalence of obesity and sedentary lifestyle (two modifiable risk factors for diabetes) was significantly higher in the diabetes belt than in the rest of the United States[5].

However, it should be noted that similar to the other analytical techniques, spatial epidemiology also has potential limitations[5,6]. First, the basic analysis approach of spatial epidemiology is based on ecological analysis design. Exposures and responses are measured only for aggregates rath than individuals. Therefore, findings from the analysis are subject to have ecological fallacy[5-8]. For example, results from an ecological analysis suggested that there was a significant correlation between increased state-level stroke prevalence and state-level stroke mortality. However, of the study states, several states that had higher state-level stroke prevalence rates did not have high stroke mortality rates, which led to a relatively weaker association than results from analyses using individual-level data[2]. Second, most spatial epidemiological studies apply age-adjusted rates to examine and map the variations in disease rates across geographic areas, such as neighborhoods, communities, districts, counties, states and countries at a global level. However, the calculation of age-adjusted rate is based on the proportion of age distributions across the geographics defined areas. If the proportions of age distributions vary widely between the comparison areas or regions, a simple weighted age-adjusted rate may be meaningless and may lead to an inappropriate comparison[5]. Third, data from disease registries, such as a small regional cancer registry, disease surveillance, or data from hospital electronic health records in a specific township is susceptible to information bias as a result of limited sources. Fourth, data protection and confidentiality should be kept in mind, specifically if mapping disease across small areas, such as small neighborhoods. It is likely that the number of persons at risk (*i.e.*, denominators) and the number of cases (*i.e.*, numerators) are too small to be used[8,9]. In the situation, a combined sample should be considered[10]. Lastly, confounding effects on the study association between exposures and outcomes should be considered and controlled appropriately in spatial epidemiological study.

In conclusion, the application of spatial epidemiology plays a pivotal role in advancing our understanding of the geographic distributions of specific disease and disease risk factors, which significantly contributes to disease control and prevention at population and community levels. However, the limitations of the study design and analysis approaches should be kept in mind when applying it in research and in public health practice.

FOOTNOTES

Author contributions: Liu L drafted the Letter; Nagar G, Diarra O, Shosanya S, Sharma G, Afesumeh D, and Krishna A critically reviewed the Letter.

Conflict-of-interest statement: The authors have no conflict of interest to disclose.

Country/Territory of origin: United States

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 August 15; 13(8): 587-667

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJD* as 4.560; IF without journal self cites: 4.450; 5-year IF: 5.370; Journal Citation Indicator: 0.62; Ranking: 62 among 146 journals in endocrinology and metabolism; and Quartile category: Q2.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE
August 15, 2022

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World J Diabetes 2022 August 15; 13(8): 587-599

DOI: 10.4239/wjd.v13.i8.587

ISSN 1948-9358 (online)

MINIREVIEWS

Diabetic kidney disease in pediatric patients: A current review

Carmen Muntean, Iuliana Magdalena Starcea, Claudia Banescu

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: He Z, China; Zavaleta MJC, Peru

Received: March 27, 2022
Peer-review started: March 27, 2022
First decision: May 30, 2022
Revised: June 13, 2022
Accepted: July 11, 2022
Article in press: July 11, 2022
Published online: August 15, 2022

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Abstract

In the last decades, a significant increase in the incidence of diabetic kidney disease (DKD) was observed concomitant with rising diabetes mellitus (DM) incidence. Kidney disease associated with DM in children and adolescents is represented by persistent albuminuria, arterial hypertension, progressive decline in estimated glomerular filtration rate to end-stage renal disease and increased cardiovascular and all-cause morbidity and mortality of these conditions. In medical practice, the common and still the "gold standard" marker for prediction and detection of diabetic kidney involvement in pediatric diabetes is represented by microalbuminuria screening even if it has low specificity to detect early stages of DKD. There are some known limitations in albuminuria value as a predictor biomarker for DKD, as not all diabetic children with microalbuminuria or macroalbuminuria will develop end-stage renal disease. As tubular damage occurs before the glomerular injury, tubular biomarkers are superior to the glomerular ones. Therefore, they may serve for early detection of DKD in both type 1 DM and type 2 DM. Conventional and new biomarkers to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies are necessary to delay the progression of kidney disease to end-stage kidney disease. New biomarkers and therapeutic strategies are discussed as timely diagnosis and therapy are critical in the pediatric diabetic population.

Key Words: Diabetes; Kidney disease; Biomarkers; Microalbuminuria; Therapy; Children

Core Tip: Several reviews in the literature contributed to the pathophysiology, diagnostics and therapeutic options for diabetic kidney disease in pediatric patients. In this review, we reported the latest data regarding novel biomarkers and methods to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies to delay the progression of kidney disease to end-stage kidney disease.

Citation: Muntean C, Starcea IM, Banescu C. Diabetic kidney disease in pediatric patients: A current review. *World J Diabetes* 2022; 13(8): 587-599
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/587.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.587

INTRODUCTION

Diabetes mellitus (DM), a chronic metabolic condition, is characterized by complete or insufficient insulin production. The main form of DM in childhood and adolescence is type 1 DM (T1DM) compared to type 2 DM (T2DM), which is more frequent in adulthood. Within the last 20 years, DM prevalence increased significantly worldwide. In the last decades, we have also assisted in an ascending trend in the prevalence of T2DM in childhood and youth because of the outbreak in juvenile obesity prevalence [1]. T1DM and T2DM have similar symptoms upon diagnosis, and both include polyuria, polydipsia and polyphagia. While obesity and insulin resistance signs (acanthosis nigricans and polycystic ovarian syndrome) are typical hallmarks of T2DM, loss of weight may be present in both types of DM[1].

Both T1DM and T2DM, with lasting inadequate glycemic control, are associated with long-term vascular complications[2] and a significant increase in mortality, especially in those who develop kidney disease[3]. While DM represents the main worldwide cause of end-stage kidney disease in adults, this is uncommon during childhood[2,3].

Although specific kidney structural changes in DM patients, namely thickening of the glomerular basement membrane and mesangial expansion, appear soon after DM onset (1.5 years to 5.0 years), they are in a clinically silent phase[4]. These structural changes of diabetic kidney injury progress at different rates among T1DM patients, and this is more evident in T2DM cases[4]. Clinical and biological abnormalities (micro/macroalbuminuria) and glomerular filtration rate (GFR) decline will develop over a longer period (10 years to 25 years)[3]. This emphasizes that diabetic kidney disease (DKD) starts early. Therefore, an early diagnosis, intensive monitoring and therapeutic interventions are necessary. Albuminuria and changes in GFR, which are late biomarkers, are the most used tools to assess kidney involvement. Diagnostic strategies for early diagnosis of kidney involvement are necessary.

There are several reviews in the literature that contributed to the pathophysiology, diagnostics and therapeutic options for DKD in pediatric patients. In this work, the state-of-the-art novel biomarkers and methods to identify diabetic children and adolescents at risk of renal complications at an early stage as well as renoprotective strategies to delay the progression of kidney disease to end-stage kidney disease was carried out.

EPIDEMIOLOGY OF DM IN CHILDREN

From 2002 to 2015 the Centers for Disease Control and Prevention reported a 4.8% increase *per* year for T1DM and a 1.9% increase *per* year for T1DM in youths aged < 20 years[5]. A very recent study, comprising six areas of the United States from 2001 to 2017, reported an important increase in estimated prevalence for both T1DM and T2DM (T1DM from 1.48 to 2.15 *per* 1000 youths < 19 years and T2DM from 0.34 to 0.67 *per* 1000 youths among those aged 10-19 years)[6]. Up-to-date research that included a large cohort of Hungarian children and teenagers during the period 2001 to 2016 (covering 16 years), showed that T1DM is still the most common type, and its prevalence is rising, with a significant male predominance (male/female ratio: 1.25). Also, there is a high prevalence of T2DM, affecting more females every year (female/male ratio: 2.86)[7]. A Danish study showed no increase in T2DM prevalence in children and adolescents[8], while in the United Kingdom a rising incidence and prevalence of T2DM have been observed in youths, especially in some ethnicities[9].

Contributing risk factors to this major increase in incidence are obesity, race, ethnicity, exposure to maternal obesity and diabetes as well as exposure to environmental contaminants[6]. There is an increased morbidity and mortality rate, mainly in T1DM and in those with early T2DM onset. According to Rhodes *et al*[10], a considerably lower life expectancy (approximately 15 years) was observed in the diabetic group compared to the general population of children without diabetes[10]. A significantly shorter life expectancy was reported in children developing T1DM before 10 years of age (loss of 17.7 years for females *vs* 14.0 years for males) compared with those diagnosed at 25-30 years

(loss of 10.0 years for females and 9.4 years for males)[11]. There is a double cardiovascular risk in pediatric diabetes that triggers early cardiovascular mortality and a four-fold higher mortality rate for all causes in youth[12]. In a nationwide Swedish study of patients with T1DM, age before 10 years at diabetes onset, was the most important risk factor for survival and cardiovascular disease (coronary heart disease and acute myocardial infarction) in their early adult years, especially in females (2-3-fold higher *vs* males)[13].

DM represents the main cause of end-stage renal disease (ESRD) worldwide in adults[14]. Diabetic nephropathy affects 20% (1 in 5) of adults with diabetes[15]. Within the pediatric population, a significant increase in the incidence of DKD was also observed, the prevalence rate being three times higher in 2013 compared to 2002 (1.16% to 3.44%)[16].

A 4-fold higher risk of kidney failure was found in a large cohort of youth with T2DM *vs* those with T1DM[17]. Also, compared with the control group, those with youth-onset T2DM had a 16-fold higher risk of a kidney disorder, a 23-fold higher risk of severe renal injury and a 39-fold increased risk of ESRD[17]. A multicenter study reported that more than a quarter (28%) of T2DM youth aged under 20 years developed microalbuminuria[18].

PATHOPHYSIOLOGY

Chronic hyperglycemia leads to the occurrence of diabetic nephropathy, retinopathy and neuropathy as well as macrovascular complications (cardiovascular disease: Stroke, coronary artery disease, peripheral vascular disease)[1,19,20]. DKD recognizes four major pathogenic mechanisms: Glomerular damage, tubular injury, inflammation and oxidative stress[21] (Figure 1). In DKD patients there are important alterations in tubules as well as in the interstitium. These findings may pave the way, or they may appear concomitant with glomerular changes[22].

This is sustained by tubular hypertrophy observed in the immediate future of hyperglycemia. Also, an increase in tubular basement membrane thickening was found even among diabetic patients with normoalbuminuria. Tubular basement membrane is one of the location of the earliest structural changes. Therefore, it may represent a better severity marker of DKD than glomerular basement membrane alteration[22]. Pathological glomerular changes in DKD are typical and consist of glomerular basement membrane thickening, podocyte foot process widening, expansion of the mesangial matrix and loss of endothelial fenestrations[23].

There is a greater risk for complication occurrence in youths with T2DM *vs* adults with T1DM and T2DM[1]. The main microvascular complication of diabetes is represented by DKD and later by diabetic nephropathy, which finally leads to ESRD. In time, with diabetes evolution, clinical and biological changes will be observed (Figure 2). DKD, one of the most important and frequent complications of DM, recognizes a wide spectrum of risk factors, some of which are modifiable. Therefore, DKD occurrence or evolution may be considerably influenced by strict control of these factors that are listed in Table 1. Children with T1DM may have damaged renal function at the disease onset as acute complications through acute kidney injury (AKI) and renal tubular damage as well as chronic complications by diabetic nephropathy development[24].

Genetic aspects

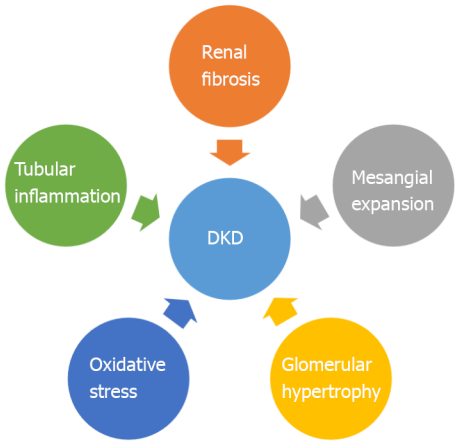
DKD is a multifactorial disorder and is influenced by genetic susceptibility, epigenetics and environmental factors (such as lifestyle, diet and medication). Also, oxidative stress, metabolic disturbance, activation of the renin-angiotensin-aldosterone system and production of inflammatory factors are involved in the development and progression of DKD[25]. Genetic and epigenetic studies were performed to understand the pathogenesis of the DKD and to identify genes that confer susceptibility to disease. Genetic studies of DKD investigated mainly the association between genomic DNA variants (for example, single nucleotide polymorphisms, copy number variants, *etc*) and clinical phenotypes of DKD in both T1DM and T2DM[26]. Epigenetic modifications (histone modifications and DNA methylation) may play a critical role in DKD as it was shown that histone acetylation and methylation are involved in the regulation of inflammation and fibrosis in DKD[27]. Epigenetics studies of DKD investigated the potentially inherited changes in gene expression that occur without changing the DNA nucleotide sequence.

Candidate gene association studies, genome-wide association studies (GWAS) and epigenome-wide association studies were performed in DKD patients[28]. A large meta-analysis study conducted by Mooyaart[29] identified 24 genetic variants in 16 genes (*EPO, APOE, APOC1, ACE, ALR2, eNOS, HSPG2, VEGF, FRMD3, GREM1, ELMO1, CCR5 and CNDP1, CARS, UNC13B* and *CPVL/CHN2*), which are the most likely to be associated with diabetic nephropathy[29]. Recently, Tziastoudi *et al*[30] conducted a systematic review and meta-analysis of genetic association studies in diabetic nephropathy in order to elucidate the contribution of genetic background in the development of this disease and observed an association with the genes revealed by Mooyaart[29] and some additional genes (*ACACB, ADIPOQ, AGT, AGTR1, AKR1B1, ATP1B2, ATP2A3, CGNL1, CNDP1, CYGB-PRCD, EDN1, ENPP1, FLT4, FTO, GLO1, HMGA2, IGF2/INS/TH* cluster, interleukin genes (*IL1B, IL8, IL10*), *KCNQ1, KNG, LOC101927627,*

Table 1 Risk factors for diabetic kidney disease development

Non-modifiable	Modifiable
Small/young age at DM onset	Poor glycemic control
Diabetes duration	Glucose variability: Hypo/hyperglycemia
Puberty	Overweight/obesity
Family history of diabetic complications and insulin resistance	Dyslipidemia
Genetic factors	High blood pressure
Race/ethnicity	Microalbuminuria
	Smoking, alcohol
	Intrauterine exposure (maternal diabetes, obesity)
	Low birth weight

DM: Diabetes mellitus.



DOI: 10.4239/wjd.v13.i8.587

Figure 1 Pathogenesis in diabetic kidney disease. DKD: Diabetic kidney disease.

MTHFR, NOS3, SETD7, SIRT1, SLC2A1, SLC2A2, SLC12A3, SLC19A3, TCF7L2, TGFB1, TIMP1, TTC39C, UNC13B, VEGFA, WTAPP1, WWC1, XYLT1)[30].

Genome-wide association studies identified about 30 genes associated with DKD (for example *ELMO1, CNDP1, FRMD3, MMP9, UMOD, SLC12A3, etc*)[25]. Epigenome-wide association studies identified several genes (for example *TRPM6, AQP9, SLC22A12, HP, HYAL2, AGTX*) that have epigenetic effects on DKD[25]. The data presented above provide further evidence for the contribution of genetic factors in DKD offering new perspectives in the discovery of new therapies for personalized medicine.

DIAGNOSIS

GFR abnormalities

Hyperfiltration, defined as an increase in GFR with more than 2 standard deviations than the mean GFR value, is related to an early increase in renal blood flow and high intraglomerular pressure[31]. In the first phases of DKD, hyperfiltration is observed in up to 40% of diabetic patients[32]. In both T1DM and T2DM, hyperfiltration has been linked to GFR loss[33,34]. Hyperfiltration was noticed more frequently in females *vs* males in both T1DM and T2DM[32,35]. The estimated GFR (eGFR) in children and adolescents with T1DM or T2DM should be screened at diagnosis and then annually[36]. These ongoing changes help us to assess DKD stages, which are presented in Table 2[20,21,37]. Normal GFR values according to child age are listed in Table 3.

Table 2 Diabetic kidney disease stages

Stage	Estimated period	Characteristics	GFR	BP	Biomarker-albuminuria	Biomarker UACR mg/mmoL
1 = hyperfiltration	From diabetes onset to 5 yr	Glomerular hyperfiltration and hypertrophy. No ultrastructure abnormality. A 20% increase in renal size. ↑Renal plasma flow	N/increased	N	Normoalbuminuria < 30 mg/g	< 2
2 = silent	From 2 yr after onset	Mild GBM thickening and interstitial expansion	N	N	Normoalbuminuria < 30 mg/g	< 3
3 = incipient	5–10 yr after onset	More significant changes *vs* stage 2. Moderate tubular and GBM thickening and variable focal mesangial sclerosis	GFR–N or mild decreased	Increasing BP; +/- hypertension	Microalbuminuria appears Albuminuria 30-300 mg/g	2-20
4 = overt	10-15 yr after onset	Marked GBM thickening and variable focal mesangial sclerosis	GFR-decreased < 60 mL/min/1.73 m^2	BP↑	Macroalbuminuria > 300 mg/g	> 20
5 = uremic		Diffuse glomerulosclerosis, ESRD	GFR-marked decreased < 15 mL/min/1.73 m^2	BP↑	Decreasing albuminuria	

UACR: Urinary albumin to creatinine ratio; GBM: Glomerular basement membrane; GFR: Glomerular filtration rate; BP: Blood pressure; ESRD: End-stage renal disease; ↑: Increase; N: Normal.

Table 3 Normal glomerular filtration rate limit at different ages according to KDOQI Guidelines[66] and Hogg *et al*[67]

Age	Gender	Normal GFR
1 wk	Males and females	41 ± 15 mL/min/1.73 m^2
2–8 wk	Males and females	66 ± 25 mL/min/1.73m^2
> 8 wk	Males and females	96 ± 22 mL/min/1.73 m^2
2–12 yr	Males and females	133 ± 27 mL/min/1.73 m^2
13–21 yr	Males	140 ± 30 mL/min/1.73m^2
13–21 yr	Females	126 ± 22 mL/min/1.73m^2

GFR: Glomerular filtration rate.

Seric and urinary biomarkers for DKD

Common markers for kidney injury are creatinine, albuminuria, cystatin C, neutrophil gelatinase-associated lipocalin and alfa-1-microglobulin in plasma and urine. Kidney function in pediatrics is assessed mainly by eGFR according to updated/bedside Schwarts equation eGFR = k × height (cm)/serum creatinine (mg/dL), k = 0.413[38].

In a recent study, 11.5% of Romanian children with T1DM had DKD, manifested as transitory microalbuminuria (7.7%) and incipient diabetic nephropathy (3.8%)[39]. In another research study, T1DM patients were found to have microalbuminuria in 30% of cases, representing the most common microvascular complication. In T1DM children the occurrence of microvascular complications was correlated with metabolic control, higher glycated hemoglobin, albuminuria, systolic blood pressure (SBP), triglycerides and total cholesterol[40].

Microvascular as well as macrovascular complications can lead to serious morbidity and mortality. Nephropathy (which is preceded by microalbuminuria), retinopathy and neuropathy represent diabetic microvascular complications[2,41]. According to the International Society for Pediatric and Adolescent Diabetes guidelines, annual microalbuminuria or urinary protein screening should start from the age of 11 years and after 2 years of diabetes evolution and then annually. It was demonstrated that persistent microalbuminuria predicts the progression to ESRD and is linked with an increased risk of macrovascular complications occurrence[41].

In T1DM pediatric patients, urine microalbumin to creatinine ratio (UACR) monitoring should start at puberty or 10 years of age (whichever is earlier), and when the child has had DM for 5 years this parameter should be checked annually. In T2DM the UACR should be checked at diagnosis and every year thereafter[36].

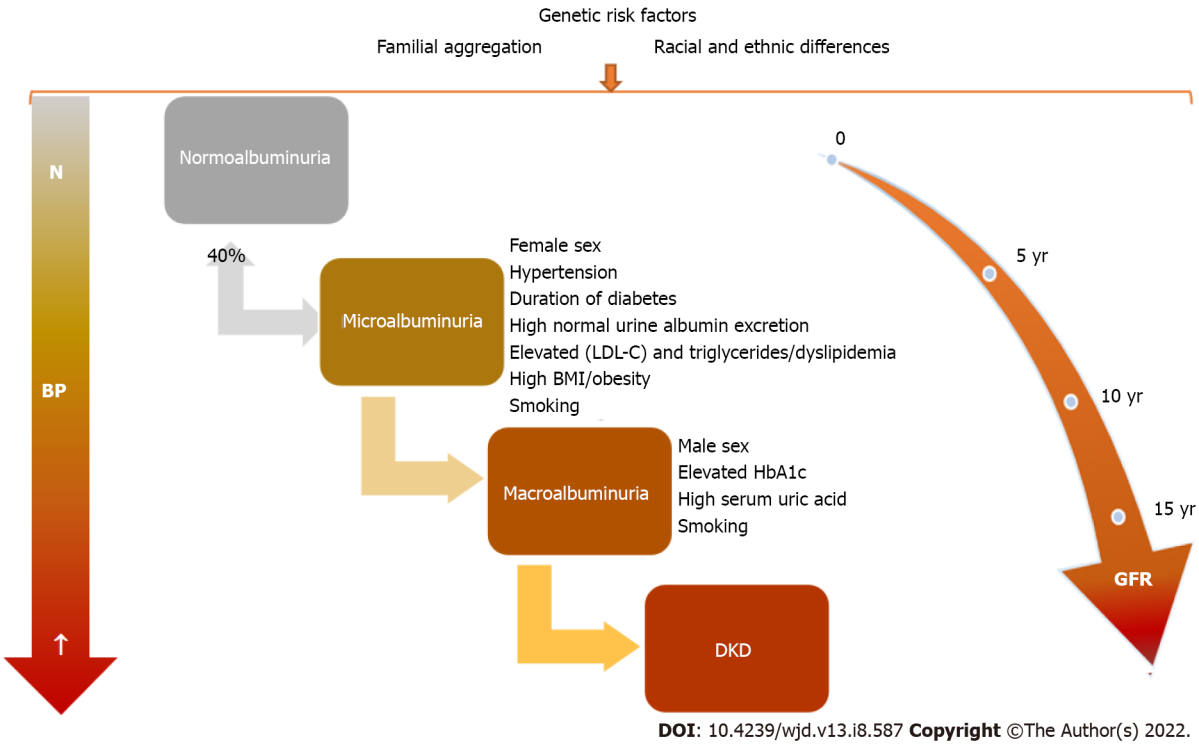


Figure 2 Changes in diabetic kidney disease: Blood pressure evolution and glomerular filtration rate decline along with albuminuria level. Influence of factors involved in diabetic kidney disease occurrence and progression. N: Normal; DKD: Diabetic kidney disease; BP: Blood pressure; GFR: Glomerular filtration rate; LDL-C: Low-density lipoprotein cholesterol; BMI: Body mass index; HbA1c: Glycated hemoglobin.

In medical practice, the common and still the "gold standard" marker for prediction and detection of diabetic kidney involvement in pediatric diabetes is represented by the microalbuminuria screening [21], even if it has a low specificity and sensitivity to detect early stages of DKD. Microalbuminuria screening should be done annually by timed overnight or 24-h urine collections (albumin excretion rate) or first-morning UACR[41].

Definitions of albuminuria and its abnormalities are based on the International Society for Pediatric and Adolescent Diabetes Clinical Practice Consensus Guidelines[37,41]. Normoalbuminuria is defined as a urine albumin level of ≤ 30 mg/L in all first-morning urine specimens, while microalbuminuria is characterized by the presence of an albumin limit of 30–300 mg or 20–200 μg/min in 24-h urine collection or a value of 30-300 mg/L in at least 2 of 3 first-morning urine specimens. Another parameter, namely UACR of 2.5–25.0 mg/mmol in males or 3.5–25.0 mg/mmol in females in at least 2 of 3 first-morning urine specimens quantifies microalbuminuria. Macroalbuminuria is defined as the presence of > 300 mg/L albumins in at least two first-morning urine specimens[37,41].

There are some limitations in albuminuria value as a biomarker for the prediction and detection of DKD, as not all diabetic children with micro- or macroalbuminuria will present a decrease in kidney function. Also, there are a lot of factors that may influence albuminuria level, UACR and eGFR: Fever, infection, diet, hydration status, hemodynamics, stress, physical activity, periods and hyperglycemia. Furthermore, a significant proportion of cases with microalbuminuria (up to 40%) may return to normoalbuminuria with strict glycemic and blood pressure (BP) control. Therefore, microalbuminuria can be transitory[21].

Microalbuminuria incidence in children with T1DM spans between 3% to 30%[37]. A cross-sectional study that involved children with T1DM reported a 25.0% frequency for microalbuminuria, while macroalbuminuria was found in 3.5% of these cases. The results of the cited study revealed a significantly higher (3 times) prevalence of microalbuminuria in T2DM (68%) compared to T1DM (24%) patients[37]. This is of particular interest given that children with T1DM are already at risk for renal complications secondary to DKD over the long term. A recent study reported early occurrence of microalbuminuria within 2 years of diagnosis of DM in 3.5% (7 of 199) of patients, whereas in 2 of those with microalbuminuria it appeared within the 1st year of diagnosis (in 7 mo)[37].

In a recent study, Hursh *et al*[24] showed that more than 64% of children hospitalized for DKD developed AKI. The same authors showed that a decreased serum bicarbonate level (< 10 mEq/L) and an increased heart rate are associated with a higher risk of severe AKI[24]. Higher morbidity and mortality rate is encountered in diabetic children that developed AKI along with a higher risk of chronic kidney disease, a finding that is particularly important in these patients who are already at risk for DKD [24].

It is already known that patients with DM may present with kidney damage (decrease in GFR) but without micro- or macroalbuminuria[42]. Therefore, other biomarkers that precede albuminuria should be considered more reliable to predict renal lesions, especially in the early stages. However, most of these biomarkers still need validation in clinical practice[43].

As tubular damage occurs before the glomerular injury, tubular biomarkers are superior to the glomerular ones, namely microalbuminuria. Therefore, they may serve for early detection of DKD in both T1DM and T2DM[44]. Tubulointerstitial damage may be suggested by the urinary albumin-to-creatinine to total protein-to-creatinine ratio of 0.40, with high sensitivity and specificity[45].

In patients without glomerular involvement, low-molecular-weight (LMW) proteinuria or non-albumin proteinuria represents an adequate marker of tubular dysfunction[46]. Urinary LMW proteins are absorbed in the proximal tubules so healthy individuals excrete up to 20 mg of LMW proteins/d in urine[46]. Alpha1 microglobulin, beta-2 microglobulins, immunoglobulin light chains, retinol-binding protein, cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-β-D-glucosaminidase, kidney injury molecule 1 and liver-type fatty acid-binding protein, *etc* are included in the LMW protein group[46]. In the early period of diabetes, an increase in urinary tubular biomarkers suggests that kidney injury is present[47].

A recent study showed the association of proximal tubule (alpha-1 microglobulin and kidney injury molecule 1) and podocyte (nephrin, vascular endothelial growth factor) damage biomarkers in T2DM even in the normoalbuminuric stage, indicating they may serve for early DKD diagnosis[47].

Urinary NGAL level increases before the onset of microalbuminuria in the very early phase of the kidney disease[48]. Alongside urinary biomarkers of tubular health (NGAL), the oxidative stress biomarker (pentosidine) may be used in the early detection of diabetic nephropathy before the microalbuminuric phase, as they correlate with albumin excretion and loss of nocturnal dipping of SBP and mean arterial BP[49].

Klotho, a transmembrane protein, is composed of a large extracellular and a small intracellular domain. Klotho is highly expressed in the renal tissue, especially in the distal tubules. The extracellular domain is cleaved by membrane proteases and discharged into the bloodstream, urine and cerebrospinal fluid as soluble klotho (s-klotho)[50,51]. A faster decline in eGFR was observed in DKD patients with low levels of serum s-klotho concentrations[52], which was opposite to the results of another study where s-klotho levels did not correlate with eGFR[50]. Bob *et al*[50] found a direct correlation of s-klotho levels with the rate of eGFR decline and with the serum levels of tubular injury marker kidney injury molecule 1[50]. A recent study found an inverse correlation between the klotho and glycated hemoglobin levels in T1DM children suggesting its possible role in chronic complications of diabetes occurrence[53].

Early stage prediction and recognition of DKD before microalbuminuria occurrence have a pivotal role in providing timely management. In this process, the assessment of more sensitive and specific biomarkers is essential. A new study showed that serum cystatin C may be used as a biomarker for DKD at an early stage in T1DM children with disease duration not exceeding 5 years before albuminuria detection[21]. The same study found a significantly lower eGFR-cystatin C value in diabetic children compared to controls. Also, significantly higher urinary cyclophilin A (CypA) and urinary CypA/creatinine ratios were found in T1DM children with microalbuminuria compared to the control group or normoalbuminuric subjects[21].

Salem *et al*[21] observed a better diagnostic value with the highest sensitivity (93.5%), specificity (71.4%) and accuracy (86.7%) to predict microalbuminuria in T1DM children by the combined use of serum cystatin C and urinary CypA than that of urinary CypA alone[21]. CypA, an endogenous cytosolic protein, is expressed mainly by the proximal tubular epithelial cells. A kidney injury is followed by an increase in urinary CypA concentration[21]. CypA level proved an encouraging biomarker for the early stage of diabetic nephropathy in adults with T2DM, and it correlates with the progression of diabetic nephropathy[54-56]. Novel biomarkers (Table 4) were proposed as early predictors of DKD[21,43].

Urinary biomarkers in DKD are crucial as they can indicate the site of injury within the nephron (structural biomarkers) as well as the loss of/reduced function of the nephron (functional biomarkers) and the main pathophysiological pathways (pathophysiological biomarkers)[57]. The proposed functional and/or structural tubular biomarkers might be valuable in the timely detection of DKD[57].

BP in diabetic children

Another important sign of diabetes-related nephropathy is BP measurement. In pediatric T2DM the guidelines recommend BP and UACR evaluation at diagnosis and annually thereafter[58].

An important and modifiable risk factor for the development of DKD is hypertension[59]. Arterial hypertension is an important and frequent risk factor for the appearance of cardiovascular disease in T1DM patients. High BP triggers the development and progression of microvascular complications, namely nephropathy, and retinopathy.

Ambulatory blood pressure measurement is superior to office BP measurements in predicting future cardiovascular events and targeting organ damage[60]. In their study, Shalaby and Shalaby[60] showed an abnormal BP profile for systolic and diastolic BP, with significant loss of nocturnal dipping. A significantly higher frequency of non-dipping patterns was observed in T1DM patients with microalbu-

Table 4 Renal biomarkers of diabetic kidney injury[21,43]

Biomarkers				
Traditional biomarkers	Traditional biomarkers of glomerular injury	Albumin/creatinine ratio eGFR	Lack of specificity and sensitivity	(1) Predict the late stages of DKD; (2) Daily variation in urine albumin/creatinine ratio; and (3) eGFR values may be affected by the patient's hemodynamics, diet and hydration status
Novel biomarkers	Glomerular biomarkers	NF-α, transferrin, Type IV collagen, L-PGDS, IgG, cerulo-plasmin, laminin, GAGs, fibronectin, podocalyxin, VEGF	Appear before microalbuminuria	Early predictor of DKD
	Tubular biomarkers	α-1-microglobulin CysC; KIM-1; NGAL; nephrin; NAG; L-FABP; VDBP; CypA; s-Klotho	Appear before/precede microalbu-minuria	(1) Are more sensitive *vs* new glomerular biomarkers; (2) Early predictors of DKD; and (3) Predictor of DKD progression
	Biomarkers of inflammation	Cytokines: TNF-α, IL-1β, IL-18, interferon gamma-IP-10, MCP-1, adiponectin, G-CSF, eotaxins, RANTES or CCL-5, orosomucoid	(1) Precede a significantly increased albuminuria; (2) Correlate positively with albumin excretion rate and intima-media thickness; and (3) May trigger direct renal injury	Predictor of DKD progression
	Biomarkers of oxidative stress	Urinary 8oHdG Pentosidine		Predict the development of DKD

L-PGDS: Lipocalin-type prostaglandin D synthase; IgG: Immunoglobulin G; GAGs: Glycosaminoglycans; CysC: Cystatin C; KIM-1: Kidney injury molecule 1; 8oHdG: 8-oxo-7,8-dihydro-2-deoxyguanosine; RANTES: Regulated on activation, normal T cell expressed and secreted; G-CSF: Granulocyte colony-stimulating factor; MCP-1: Monocyte chemoattractant protein 1; IP-10: Induced protein-10; TNF-α: Tumor necrosis factor α; IL: Interleukin; CypA: Cyclophilin A; VDBP: Vitamin D-binding protein; L-FABP: Liver-type fatty-acid binding protein; NAG: N-acetyl-β-D-glucosaminidase; NGAL: Neutrophil gelatinase-associated lipocalin; DKD: Diabetic kidney disease; eGFR: Estimated glomerular filtration rate; VEGF: Vascular endothelial growth factor; CCL-5: Chemokine ligand 5T.

minuria[60].

A recent study that comprises 3529 children and adolescents with T1DM revealed impaired BP regulation with elevated systolic BP, nocturnal diastolic BP, mean arterial pressure and diastolic dipping but lower nocturnal systolic dipping[61]. Lurbe *et al*[62] showed that an increase in nocturnal SBP precedes microalbuminuria occurrence within T1DM children[62].

The non-dipper pattern for SBD is one of the earliest abnormalities in the BP profile detected for children with T1DM. Also, non-dipping status has been associated with kidney damage (renal morphological changes) and hyperfiltration in adolescents with T1DM[63]. Also, the non-dipping status seems to be an early predictor of later nephropathy[63].

Teenagers with T1DM are at risk for hyperfiltration and higher UACR (urinary albumin-to-creatinine ratio), which are biomarkers for early/ incipient nephropathy[35]. A recent meta-analysis found that almost 25% of T2DM patients have arterial hypertension, the male sex being more frequently affected, and that 1 in 4 or 5 children have albuminuria[58].

Mamilly *et al*[49] found an increased urinary NGAL/creatinine (a marker of tubular injury) and pentosidine/creatinine (a marker of oxidative stress) in subjects with T1DM compared to controls even in the absence of microalbuminuria[49]. The same study reported a high incidence of abnormal BP dipping, which is important because dipping abnormalities may serve as a predictor for vascular complications, especially kidney injury in diabetic individuals[49]. The same study proved that urine NGAL correlates with loss of nocturnal dipping of SBP[49].

Based on these data, ambulatory blood pressure measurement represents the gold standard to assess BP regulation and should be used in all diabetic patients for timely therapeutic intervention to prevent renal and cardiovascular diabetic complications later in life.

PROPHYLACTIC AND THERAPEUTIC STRATEGIES FOR DKD

The well-known strategies, namely rigorous glycemic control, strict BP control and modulation of obesity, still represent the most important tools to prevent and slow down the progression of diabetic nephropathy/the deterioration of renal function. These therapies proved to be effective mainly by targeting the modifiable risk factors for diabetic nephropathy, which are listed in Table 1.

A recent systematic review confirmed that early high doses of vitamin D supplementation in combination with renin-angiotensin-aldosterone system blockers may slow the onset or progression of

Table 5 Common and new therapeutic strategies in diabetic kidney disease

Therapy	Drug class	Aim	Mechanism of action	DKD result/effect	Dose adjustment to eGFR (mL/min/1.73 m²)
Conventional therapies					
Strict glycemic control (Insulin)	-	HbA1c < 7%	(1) Reduces the risk of microalbuminuria; and (2) Reduces progression of microalbuminuria to macroalbuminuria	Delay DKD progression/risk	GFR = 10–50: Reduce the dose to 75%; GFR < 10: Reduce dose to 50%
Dietary protein/phosphate restriction	-	↓High protein intake	(1) Reduces hyperfiltration; and (2) Slows down/delays the loss of function or progression of diabetic nephropathy in T1DM and T2DM	Lower DKD risk	No restriction. CKD stage 3: 100%-140% of the DRI. CKD stage 4-5: 100%-120% of the DRI
Weight loss, increased physical activity	-		(1) Reduces hyperfiltration; and (2) Reduces albuminuria, especially in moderate/severe obesity	Lower DKD risk	No
Antihypertensive therapy	(1) ACEI/ARB/calcium-channel blockers; and (2) ACEI/ARB + calcium-channel blockers	Control of BP	(1) Reduces albuminuria and delays the onset of DN; (2) Prevents progression of DN in microalbuminuric patients; and (3) Reduces the frequency of microalbuminuria in hypertensive normoalbuminuric cases	Delay DKD progression	ARB, calcium channel blockers: No adjustment ACEI: GFR 30-60: Reduce dose to 50%; GFR < 30: Stop
Treatment of Dyslipidaemia	(1) Atorvastatin; (2) Fluvastatin; and (3) Osuvastatin	Reduce LDL-C	Reduce albuminuria in patients with DKD receiving RAAS blockers	Reduces CV disease/risk	No
Psychological Intervention	(1) Family therapy; (2) Cognitive behavioral therapy; (3) Motivational interviewing; (4) Counselling; (5) Mentoring; and (6) Peer support	Reduce depression	Follow lifestyle adjustment regimens and achieve optimal glucose levels	Delay DKD progression	No
Novel therapies					
Vitamin D analogues	Paricalcitol. Calcitriol		(1) Ameliorates nephropathy by reducing the albuminuria; and (2) Prevent glomerulosclerosis	Delay DKD progression	No
Vitamin D metabolites			Inhibit RAAS and prevent glomerulosclerosis	Delay DKD progression/risk	No
Uric acid antagonist	Allopurinol	Uric acid antagonist/xanthine oxidase inhibitor	(1) Reduces urinary TGF-β1 in diabetic nephropathy; (2) Reduces albuminuria in T2DM; and (3) Improves endothelial dysfunction	Delay DKD risk/progression	GFR > 50: No adjustment. GFR 30-50: Reduce dose by 50%. GFR < 10: Reduce dose to 30%, longer interval
Renin inhibitor	Aliskiren	Block RAAS cascade	Reduces albuminuria and serves as an antihypertensive in T2DM	Delay DKD progression	No
Endothelin antagonist or I inhibitor ETA receptor antagonist	Atransetan, avosentan, sparsentan (irbesartan + ETA)		(1) Reduces residual albuminuria in type 2 diabetic nephropathy; (2) Reduces proteinuria in T2DM patients and nephropathy; and (3) Significant proteinuria reduction	Delay/slow DKD progression	Yes
MRA Mineralocorticoid Receptor Antagonists	Spironolactone = nonselective MRA. Eplerenone	↑Natriuresis	Reduce albuminuria and blood pressure in patients with DN when added to a RAAS inhibitor	Delay DKD risk/progression	GFR > 50: No dose adjustment. GFR 30-50: Reduce dose to 25%, once daily.

					GFR < 10: No use
SGLT2 inhibitors	Empagliflozin, canagliflozin	Glucose-lowering	(1) Improves glycaemic control, reduces fasting blood glucose and HbA1c by increasing urinary glucose excretion; and (2) Reduces the reabsorption of sodium	Delay DKD progression, reduces blood pressure	No
GLP-1 agonist	Liraglutide, semaglutide	Stimulates insulin secretion, ↑satiety	Improves glycaemic control	Delay DKD risk/progression	No
	Exenatide, lixisenatide	Stimulates insulin secretion	Improves glycaemic control	Delay DKD risk/progression	Caution in CrCl < 50 mL/min
DDP-4 inhibitors	Linagliptin, saxagliptin, vildagliptin	Glucose-lowering-preserve the glucagon-like peptide effect	Reduce albuminuria in macroalbuminuric T2DM patients	Delay DKD risk/progression	eGFR < 50 mL/min: Reduce dose by 50%; eGFR < 30 mL/min: Reduce dose by 75%
TZD Thiazolidine-diones	Rosiglitazone. Pioglitazone	↓Hepatic glucose production activate peroxisome proliferator-activated receptor-γ to increase tissue insulin sensitivity	(1) Reduce albuminuria in macroalbuminuric T2DM patients; and (2) Lower microalbuminuria and proteinuria	Delay DKD risk/progression	No
Aldosterone synthase (CYP11B2) inhibition		Decrease in plasma aldosterone levels		Delay DKD risk/progression	NL
Anti-inflammatory Compounds					
CCR2 Antagonists		Emapticap pegol (NOX-E36), CCX-140	Reduces UACR and HbA1c	In T2DM-delay DKD, DN risk/progression	NL
VAP-1 inhibitors	An adhesion molecule for lymphocytes, regulating leukocyte migration into inflamed tissue	ASP-8232	Reduces albuminuria in T2DM in CKD	Delay DKD risk/progression	NL

ETA: Endothelin type A; T2DM: Type 2 diabetes mellitus; DKD: Diabetic kidney disease; UACR: Urine microalbumin to creatinine ratio; HbA1c: Glycated hemoglobin; GFR: Glomerular filtration rate; RAAS: Renin-angiotensin-aldosterone system; eGFR: Estimated glomerular filtration rate; ↓: Decreased; T1DM: Type 1 diabetes mellitus; CKD: Chronic kidney disease; DRI: Dietary reference intake; ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin II receptor blocker; BP: Blood pressure; DN: Diabetic nephropathy; LDL-C: Low-density lipoprotein cholesterol; CV: Cardiovascular; TGF-1: Transforming growth factor 1; MRA: Mineralocorticoid receptor antagonists; SGLT-2: Sodium-glucose cotransporter-2; GLP-1: Glucagon-like peptide 1; CrCl: Creatinine clearance; DPP-4: Dipeptidyl peptidase 4; TZD: Thiazolidinediones; NL: Not listed; CCR2: Chemokine receptor 2; VAP-1: Vascular adhesion protein 1.

DKD[64]. Standard and some novel proposed therapies in early-stage or late-stage development of diabetic nephropathy are presented in Table 5[20,64,65].

CONCLUSION

DKD, the most significant and frequent burden of this metabolic disorder, is still discovered late as microalbuminuria is the most used biomarker for predicting kidney involvement. Novel biomarkers are valuable tools in the detection of kidney damage in the early phases as well as reliable predictors for DKD progression. Therefore, effective therapies may be proposed. Early stage prediction and recognition of DKD in children and adolescents before microalbuminuria occurrence have a pivotal role in preventing the development of and/or progression to irreversible kidney damage and to provide timely management and appropriate treatment by using conventional and novel therapies that may slow the onset or progression of DKD.

FOOTNOTES

Author contributions: All authors contributed equally to this work; Muntean C and Banescu C contributed to conception and design of the work, interpreting the relevant literature and drafting the manuscript; Muntean C,

Banescu C and Starcea IM performed the research of the literature; Muntean C and Starcea IM made critical revisions of the manuscript; all authors have read and approved the final version of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 600-612

DOI: 10.4239/wjd.v13.i8.600

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Clopidogrel delays and can reverse diabetic nephropathy pathogenesis in type 2 diabetic *db/db* mice

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Balbaa ME, Egypt; Saengboonmee C, Thailand

Received: October 18, 2021
Peer-review started: October 18, 2021
First decision: December 27, 2021
Revised: January 26, 2022
Accepted: June 27, 2022
Article in press: June 27, 2022
Published online: August 15, 2022

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Abstract

BACKGROUND

Diabetic nephropathy (DN) is the principal cause of end-stage renal disease. Previous studies have shown that clopidogrel can prevent the early progression of renal injury.

AIM

To elucidate whether clopidogrel is beneficial against DN by using a *db/db* mouse model.

METHODS

db/db mice with a higher urinary albumin/creatinine ratio (ACR) relative to age- and sex-matched wild-type control mice were randomly allocated to clopidogrel and vehicle treatment groups. Clopidogrel was administered at doses of 5, 10, and 20 mg/kg by gavage for 12 wk. Body mass, blood glucose level, and urinary creatinine and albumin concentrations in each group were measured before and after the intervention. Renal fibrosis was evaluated using periodic acid-Schiff and Masson's trichrome staining. The renal protein expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and F4/80 was assessed using immunohistochemistry. Urinary TNF-α, monocyte chemoattractant protein-1 (MCP-1), and IL-6 levels were analyzed using enzyme-linked immunosorbent assay; *TNF-α* and *IL-1β* mRNA expression was measured using real-time quantitative polymerase chain reaction. The protein expression of fibronectin (FN) and collagen I was assessed using immunohistochemistry.

RESULTS

Clopidogrel treatment did not affect the body mass or blood glucose level of the *db/db* mice; however, it increased bleeding time and reduced urinary ACR in a

dose-dependent manner. Immunohistochemical staining revealed an amelioration of renal fibrosis, significantly lower deposition of FN and collagen I, and significantly lower expression of the proinflammatory cytokines TNF-α and IL-1β and lower levels of urinary TNF-α and MCP-1 in the clopidogrel-treated *db/db* mice ($P < 0.05$). Furthermore, clopidogrel significantly reduced macrophage infiltration into the glomeruli of the *db/db* mice.

CONCLUSION

Clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Key Words: Diabetes; Clopidogrel; Inflammation; Fibronectin; Diabetic nephropathy

Core Tip: Diabetic nephropathy is the most common microvascular inflammatory disease among the diabetic complications. Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in diseases such as acute myocardial infarction, diabetes, and acute ischemic cerebral infarction. In this study, we aimed to determine whether treatment with clopidogrel has a preventive or therapeutic effect in the kidneys of obese, type 2 diabetic *db/db* mice. In this experiment, we demonstrated that clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Citation: Li HQ, Liu N, Zheng ZY, Teng HL, Pei J. Clopidogrel delays and can reverse diabetic nephropathy pathogenesis in type 2 diabetic *db/db* mice. *World J Diabetes* 2022; 13(8): 600-612
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/600.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.600

INTRODUCTION

Diabetes is a major public health issue, affecting more than 400 million people worldwide[1]. Type 2 diabetes is a highly prevalent condition that is associated with major vascular, renal, and neurologic complications. Diabetic nephropathy (DN) is the most common microvascular inflammatory disease among diabetes complications. Proteinuria is a hallmark of early DN, and the associated morphological abnormalities include glomerular hypertrophy, thickening of the glomerular basement membrane, expansion of the mesangial matrix, and renal tubular injury. Renal changes in the later phases include glomerulosclerosis and tubulointerstitial fibrosis[2-4].

The mechanism of DN is complex. A persistently high glucose level is considered the principal risk factor for DN; however, other factors, such as abnormal renal hemodynamics, may also be involved in the development of DN[5]. Hyperglycemia leads to the expression of proinflammatory mediators (chemokines and cytokines) in injured glomerular and tubular cells, which contributes to renal damage through various mechanisms, including mesangial proliferation, podocyte/tubular damage, and leukocyte infiltration[6,7]. Leukocytic interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α promote the release of vascular endothelial factors, stimulate the proliferation of glomerular mesangial cells, increase the permeability of the endothelium, and promote the synthesis and release of superoxide and proteolytic enzymes, which eventually cause renal structural remodeling and dysfunction[8,9]. An increasing amount of evidence indicates that diabetes-associated vascular and renal inflammation is likely to be associated with high platelet reactivity, which would contribute to high atherothrombotic risk[10,11].

Clopidogrel is an anti-platelet aggregation drug that with a pyridine-based structure. It can specifically and irreversibly bind to platelet P2RY12 purinergic receptors, which inhibits ADP-mediated platelet activation and aggregation. Clopidogrel not only inhibits platelet aggregation but also inflammatory responses in a platelet activation-dependent or independent manner[12,13]. Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in conditions such as acute myocardial infarction, diabetes, and acute ischemic cerebral infarction[14,15]. Lower expression levels of IL-6, TNF-α, and transforming growth factor-β1; lower matrix metalloproteinase (MMP)-2 and MMP-9 activity; and stabilization of the extracellular matrix (ECM) in clopidogrel-treated mice with hyperlipidemia and acute myocardial infarction reflected the protective effect of the drug[16]. We have previously shown that clopidogrel significantly reduced renal collagen and

fibronectin (FN) expression and thus ameliorated diabetes-induced renal fibrosis in a streptozotocin-induced murine model of type 1 diabetes[17]. However, because 80%-90% of the population with diabetes has type 2 disease[1], in the present study, we aimed to determine whether clopidogrel treatment has a preventive or therapeutic effect in the kidneys of obese type 2 diabetic *db*/*db* mice, a widely used mouse model of type 2 diabetes for DN investigations[18].

MATERIALS AND METHODS

Experimental animals, groups, and drug administration

db/db mice used in the present study were leptin receptor (Lepr) knockout mice with the C57BLKS background developed by GemPharmatech Co., Ltd. by using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique, (https://www.gempharmatech.us/en/strain/detail/?strain_id=3913). This mouse line has features similar to that from the Jackson Laboratory (https://www.jax.org/strain/000642): Significant increases in the body weight starting at 4 wk, hyperglycemia (6-h fasting blood glucose and hemoglobin A1c) at 8 wk, insulinemia (> 3-fold) at 8 wk, and hyperlipidemia (cholesterol, triglyceride, and low-density lipoprotein) along with the early onset of renal dysfunction (indicated by significantly increased microalbumin level in 24-h urine analysis) at 12 wk of age. Therefore, this mouse line has been widely used as a model for studies on type 2 diabetes[19, 20], including for the studies on DN of type 2 diabetes[18,21].

Eight-week-old, specific pathogen-free male C57BLKS *db*/*db* diabetic mice (n = 24) and age-matched C57BLKS [wild-type (WT)] mice (n = 6) were purchased from the Model Animal Research Center of Nanjing University (Cat. No. T002407, GemPharmatech Co., Ltd., Nanjing, China). All animal experiments were approved by the ethics committee of the First Hospital of Jilin University and conformed to the internationally accepted principles for the care and use of laboratory animals. All the mice were adoptively fed until 12 wk of age when these *db/db* mice should show typical DN, defined as the baseline. Then the *db*/*db* mice were randomly assigned to vehicle or low-dose (5 mg/kg), medium-dose (10 mg/kg), or high-dose (20 mg/kg) clopidogrel groups. The mice were administered clopidogrel or vehicle daily by gavage for 3 mo. At the end of the experiment, the animals were intraperitoneally anesthetized with tribromoethanol (350 mg/kg) and sacrificed to collect blood and kidneys for subsequent experiments.

Sample collection

Blood glucose levels were measured at regular intervals by using samples collected from a tail vein. Twenty-four-hour urine collections were performed before and after 3 mo of treatment with the animals in metabolic cages, and the 24-h urine albumin output was measured using a mouse microalbuminuria enzyme-linked immunosorbent assay (ELISA) kit (Beijing XinQuan Tech Company, XQ-EN20536). The urine creatinine output was measured using a creatinine test kit (Nanjing Jiancheng Bioengineering Institute, C011-1-1).

Blood clotting time

Blood clotting time was measured using the mouse tail-vein bleeding assay. Briefly, the mouse's tail was cut 1-2 mm from the tip, where its diameter was approximately 1 mm, and then, it was immediately dipped into a 50-mL tube filled with saline at 37 °C. The bleeding time was recorded over a period of up to 5 min, as in our previous study[17].

Histology and immunohistochemical staining

The collected kidneys were fixed in 10% formalin, dehydrated in a graded series of alcohol, cleaned with xylene, embedded in paraffin, and sectioned at a thickness of 5 μm. Periodic acid-Schiff and Masson staining was performed to facilitate the examination of the glomerular basement membrane and mesangial matrix of the kidneys. Immunohistochemistry was used to assess the expression of TNF-α (Abcam, ab220210), IL-1β (Cell Signaling Technologies, #12242), F4/80 (Abcam, ab100790), FN (Santa Cruz Biotechnology, SC-8422), and collagen I (Abcam, ab253113) in the kidney sections. After 3,3′-diaminobenzidine staining, cells that were positive for target protein expression stained brown-yellow, whereas the cells without the said expression were unstained. The percentage of each area that was stained was quantified using the Image-Pro Plus software.

Measurement of TNF-α, MCP-1, and IL-6 levels in the urine

Urinary TNF-α, MCP-1, and IL-6 levels were measured using a mouse TNF-α precoated ELISA kit (DAKEWE, 1217202), mouse MCP-1 precoated ELISA kit (DAKEWE, 1217392), and mouse IL-6 precoated ELISA kit (DAKEWE, 1210602), respectively. These concentrations were normalized to the urinary creatinine concentrations.

RNA isolation and real-time polymerase chain reaction

RNA was extracted from tissues by using Trizol and reverse-transcribed into cDNA. Real-time polymerase chain reaction (PCR) was performed using SYBR Green chemistry, with *β-actin* as the reference gene. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. The primer sequences used were as follows: *TNF-α* (F): TATAAAGCGGCCGTCTGCAC, *TNF-α* (R): TCTTCTGCCAGTTCCACGTC; *IL-1β* (F): TTGACGGACCCCAAAAGATG, *IL-1β* (R): AGAAGGTGCTCATGTCCTCA; and *β-actin* (F): CCCTGTATGCCTCTGGTCGT, *β-actin* (R): CGTGGGGTGAAGCTGTAGCCACG.

Statistical analysis

Data are presented as mean ± SD values for $n = 6$ mice per group. Multiple comparisons of data were performed using one-way analysis of variance, with Tukey's test for *post-hoc* pairwise comparisons. All statistical analyses were conducted using GraphPad Prism 5, and $P < 0.05$ was considered to represent statistical significance.

RESULTS

Effects of the db/db genotype and clopidogrel on the body mass, blood glucose level, and bleeding time of the mice

At the age of 12 wk, the *db/db* mice were significantly heavier and had higher fasting blood glucose levels compared to the WT mice (Figures 1A and B). Their animals' body mass slightly increased with age thereafter, whereas their fasting blood glucose levels did not significantly change during the 3-mo treatment period. Because *db/db* mice start showing typical DN at 12 wk of age, we started to administer clopidogrel from this age with one of three doses (5, 10, or 20 mg/kg), to observe the drug's therapeutic effects or delay DN progression. First, the bleeding times of the WT and diabetic mice that had or had not been administered clopidogrel were measured (Figure 1C). There was no difference between diabetic mice and WT nondiabetic mice at the baseline level, and treatment of the diabetic mice with clopidogrel increased the bleeding time in a dose-dependent manner during the 3-mo treatment. Furthermore, clopidogrel treatment did not affect the body mass or blood glucose levels of the diabetic mice during the experimental period (Figures 1A and B).

Clopidogrel ameliorated diabetes-associated renal dysfunction, glomerular sclerosis, and collagen fiber deposition in the mice

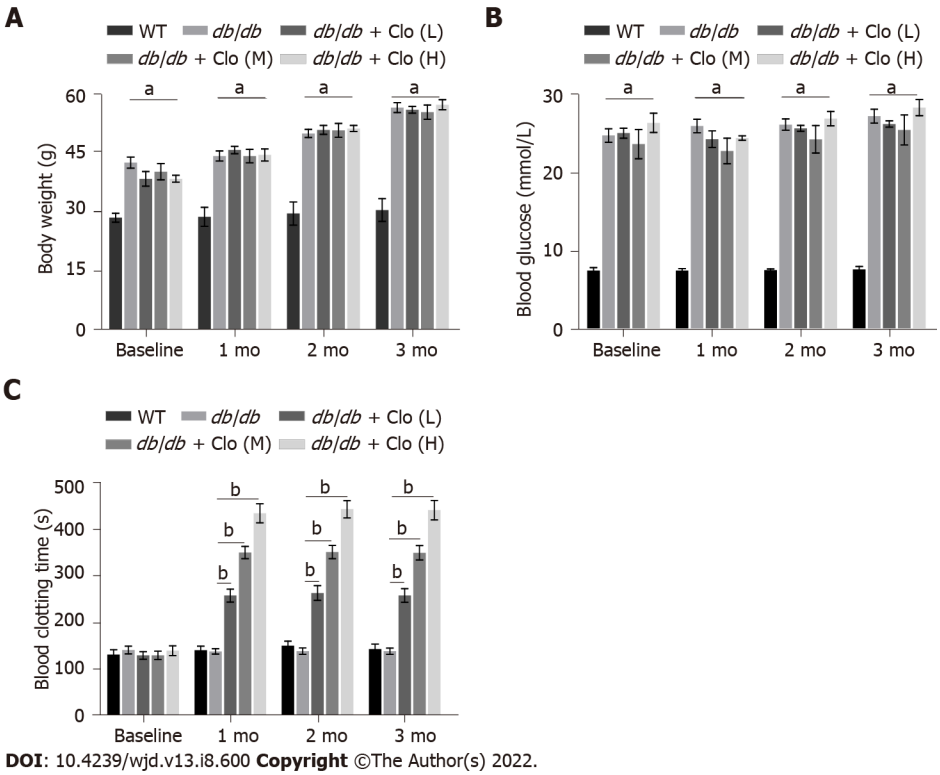
Clopidogrel administration for 3 mo reduced the urinary albumin/creatinine ratio (ACR) of the diabetic mice, especially in the high-dose group, which implies an improvement in the glomerular filtration rate (Figure 2A). The kidneys of the WT mice did not show any obvious pathology, whereas those of the *db/db* mice showed larger renal glomeruli, thickening of the substrate membranes, hyperplasia of mesangial cells, and an expansion of the ECM. These histopathological changes were markedly ameliorated by clopidogrel administration (Figure 2B). Masson's trichrome staining showed greater collagen deposition in the *db/db* mice; however, this was less severe in the mice administered clopidogrel (Figure 2B).

Potential mechanisms for the beneficial effects of clopidogrel: A reduction in the renal accumulation of ECM components responsible for fibrosis

It has been shown that in the early stages of DN, there is a significant increase in the deposition of collagen, FN, and laminin in the glomerular basement membrane and ECM in glomerular mesangial cells[22]. We assessed the protein expression of FN and collagen type I in the kidney cortex of the mice by immunohistochemical staining (Figure 3). No significant FN or collagen I protein expression was identified in the renal glomerulus of WT mice (Figure 3), and there was only a low level of expression around the blood vessels (data not shown). In contrast, there was an excessive deposition of both FN and collagen I in the glomerular membranes and mesangium of the *db/db* mice (Figure 3). However, the accumulation of both proteins was significantly lower in *db/db* mice that had been administered clopidogrel for 3 mo (Figure 3). These data imply that clopidogrel reduced collagen synthesis and ECM deposition and inhibited fibrosis in the kidneys of the *db/db* mice.

Potential mechanisms for the beneficial effects of clopidogrel: Inhibition of the diabetes-associated renal inflammatory response and macrophage infiltration

We next measured the mRNA expression of *TNF-α* and *IL-1β* by using real-time PCR, which showed that both mRNA expression levels were significantly lower in the clopidogrel treatment group (Figure 4A). Furthermore, immunohistochemical staining of kidney sections showed that TNF-α and IL-1β were expressed at low levels in the kidneys of the WT mice. However, there was higher TNF-α expression principally in the renal tubules and higher IL-1β expression in both the glomeruli and renal tubules of the *db/db* mice (Figure 4B). Semi-quantitative analysis showed significantly higher protein



DOI: 10.4239/wjd.v13.i8.600

Figure 1 Effects of clopidogrel on body mass, 6-h fasting blood glucose level in mice, and the bleeding time of mice. A: Body mass; B: Blood glucose; C: Blood clotting time. Data are shown as mean ± SD values of $n = 6$ mice per group. $^{a}P < 0.05$ *vs* wild type mice. $^{b}P < 0.05$ *vs* untreated *db/db* mice. WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel; Clo (L, M, H): Clopidogrel at 5, 10, or 20 mg/kg, respectively.

expression of TNF-α and IL-1β in the kidneys of diabetic mice. Nevertheless, clopidogrel significantly reduced the expression of these renal inflammatory cytokines (Figure 4C).

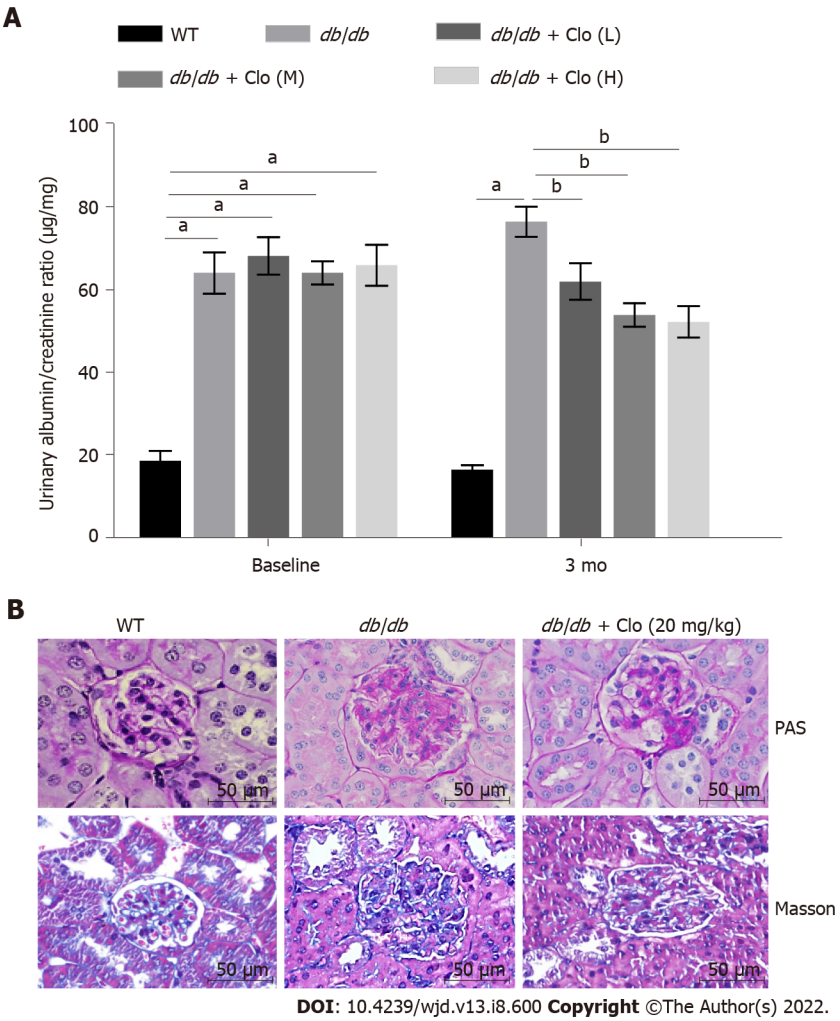
We also examined these inflammatory cytokine levels in the urine, which may be indirect indicators of systemic inflammation. Furthermore, considering that MCP-1 is a potent chemokine in the recruitment of macrophages[23], urinary TNF-α, IL-6, and MCP-1 levels were measured by ELISA (Figure 5). The results showed that the development of diabetes significantly increased urinary TNF-α, IL-6, and MCP-1 levels. However, clopidogrel reduced the levels of urinary TNF-α and MCP-1 (Figures 5A and C), and urinary IL-6 level in *db/db* mice had a slight decrease (Figure 5B). These results indicate that clopidogrel can inhibit the diabetes-associated renal inflammatory response and probably systemic inflammation.

Macrophages, lymphocytes, and mast cells secrete large amounts of proinflammatory mediators and cytokines in the diabetic kidney, which directly or indirectly induce renal damage and accelerate local fibrosis[24,25]. F4/80 is a macrophage marker expressed on the surface of these cells in mice. Consistent with the urinary MCP-1 results, there was no obvious expression of F4/80 in normal mouse kidney tissue (Figure 6). On the other hand, macrophage infiltration was apparent around the glomeruli in diabetic mice. Furthermore, the number of macrophages at this location was significantly high in *db/db* mice; this number was significantly reduced by clopidogrel treatment (Figure 6). These results suggest that clopidogrel reduces macrophage infiltration and inhibits cytokine secretion, thereby reducing damage to the kidneys caused by the inflammatory response.

DISCUSSION

Clopidogrel is an antagonist of the P2RY12 receptor, which is expressed on the surfaces of platelets. It not only inhibits platelet aggregation but also reduces ventricular inflammation and fibrosis in animal models[26-28]. Consistent with this, in this study, we found that clopidogrel administration for 3 mo delayed or prevented the progression of DN in *db/db* diabetic mice. These beneficial effects of clopidogrel in *db/db* mice appear to be mediated by inhibition of the renal deposition of collagen, probably through suppression of macrophage infiltration into the kidney and thus a reduction in proinflammatory cytokine secretion, as illustrated in Figure 7.

We have also shown that clopidogrel administration does not significantly affect the body mass or blood glucose level of *db/db* diabetic mice, which implies that the dose and duration of clopidogrel administration used in these mice were safe, without evidence of potential side-effects. Furthermore,



DOI: 10.4239/wjd.v13.i8.600

Figure 2 Clopidogrel improves the kidney function of *db*/*db* mice. Data are shown as mean ± SD values of $n = 6$ mice per group. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. A: Urinary albumin/creatinine ratio. Mice at 12 wk of age were defined as the baseline; B: Periodic acid-Schiff staining of kidney sections for glycogen content and Masson's trichrome staining for collagenous connective tissue fibers. Magnification: × 400. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel; Clo (L, M, H): Clopidogrel at 5, 10, or 20 mg/kg, respectively.

these beneficial effects of the drug against DN development in these mice were independent of glycemic control. There have been few previous studies on the effects of clopidogrel on blood glucose. However, in one study, patients with type 2 diabetes were treated daily with one 70 mg clopidogrel tablet for 2 mo, which improved their fasting blood glucose level (from 9.7 ± 0.7 mmol/L to 7.5 ± 0.5 mmol/L). Therefore, the potential hypoglycemic effect of clopidogrel requires further investigation.

In addition, although clopidogrel is an anti-platelet aggregation drug, its preventive or therapeutic effect on the development of DN in *db*/*db* mice does not seem to be predominantly related to its effect on platelet aggregation. We believe this is because the *db/db* mice did not demonstrate shorter bleeding times than those noted in the WT mice before or after 3 mo of clopidogrel administration (Figure 1C). However, clopidogrel increased the bleeding times of the *db/db* mice in a dose-dependent manner (Figure 1C) during the 3-mo treatment period, parallel to the dose-dependent effects of improvement in renal function (Figure 2A).

The urinary albumin concentration is closely related to the progression of glomerular lesions and kidney damage, and this was significantly reduced by clopidogrel administration in *db*/*db* mice to levels that were lower than those prior to treatment (Figure 2A). Inflammation plays an important role in the development and progression of DN, and this involves the production of chemokines and proinflammatory cytokines, infiltration of immune cells into the kidney, formation of immune complexes, and complement activation[29]. Chronic diabetic hyperglycemia causes an increase in the circulating concentration of advanced glycation end-products, which induce macrophage migration through advanced glycation end product receptor-mediated activation of the nuclear factor (NF)-κB inflammatory pathway[30,31]. Furthermore, the production of the proinflammatory cytokines TNF-α and IL-1β is induced by NF-κB activation. TNF-α promotes inflammatory cell aggregation and adhesion, microvascular dilation, vascular permeability, and exacerbation of the inflammatory response, thereby contributing to glomerular tissue damage. Therefore, the serum TNF-α concentration is considered a

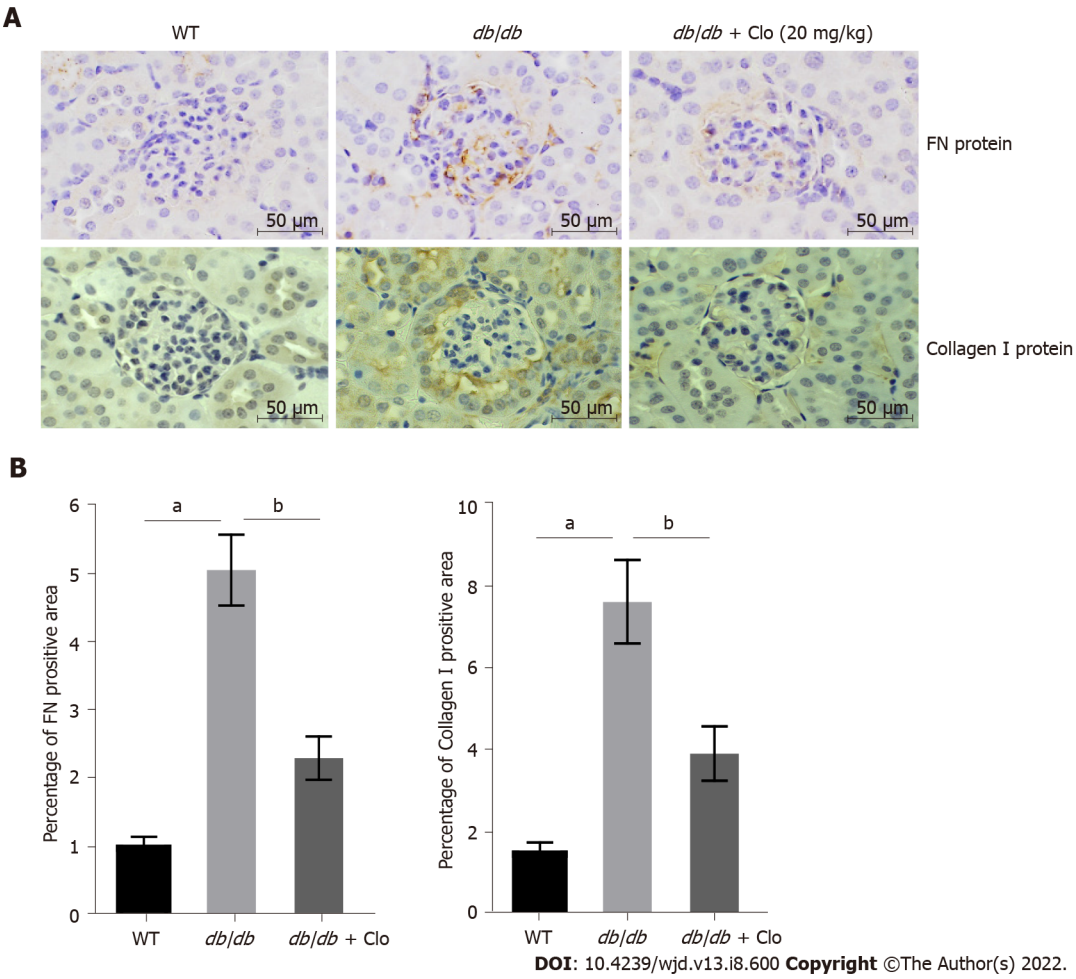
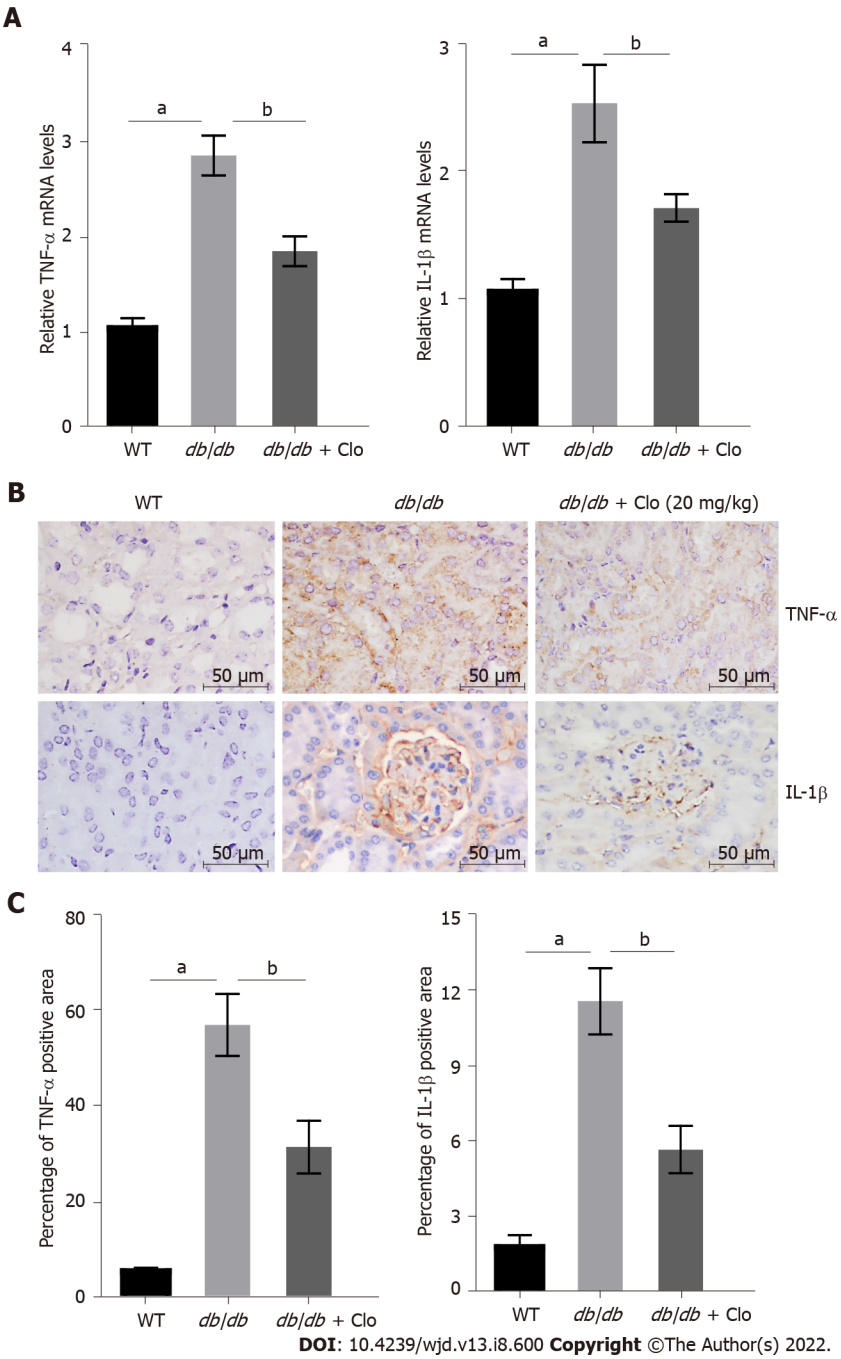


Figure 3 Clopidogrel reduces the production of fibronectin and collagen I in the kidneys of *db/db* mice. A: Representative immunohistochemical staining. Magnification: × 400; B: Quantitative analysis of fibronectin protein and collagen I protein. Data are shown as mean ± SD values of $n = 6$ mice per group. $^{a}P < 0.05$ *vs* wild type mice; $^{b}P < 0.05$ *vs db/db* mice. FN: Fibronectin; WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel.

predictor of disease progression[9,32]. IL-1β stimulates the proliferation of glomerular cells in the kidneys, promotes the production of ECM, and accelerates the process of renal fibrosis[8,33]. Consistent with this, we found significantly higher expression of both TNF-α and IL-1β in the kidneys and urine of *db/db* mice. However, treatment with clopidogrel significantly reduced the expression of both cytokines, which implies that the drug has an anti-inflammatory effect in the kidneys of diabetic mice.

Macrophages are the principal type of immune cells that promote kidney damage in diabetes[34,35]. F4/80 is a macrophage-specific antigen that participates in the maturation and activation of this cell type. Consistent with the increased urinary level of MCP-1, which is a potent chemokine in macrophages, we found a significant increase in macrophage infiltration, mirrored by F4/80-positive staining (Figure 6), in the kidneys of the *db/db* mice. However, the number of macrophages in the kidneys of the clopidogrel-treated mice was significantly lower. Chronic renal inflammation causes glomerular membrane cells to produce large amounts of type I and type IV collagen and FN, which leads to thickening of the glomerular basement membrane and ECM accumulation, ultimately resulting in glomerulosclerosis[36,37]. We also found higher expression of collagen I and FN in the kidneys of the *db/db* mice. However, this was much lower in the kidneys of mice that had been treated with clopidogrel for 3 mo.

Thus, as illustrated in Figure 7, the results of the present study suggest that clopidogrel administration for 3 mo to *db/db* mice, which had significant renal dysfunction (high urinary ACR), delayed the progression of DN and possibly improved renal function. This effect is accompanied by an amelioration of features of the renal pathology of DN, including lower renal accumulation of ECM components, such as collagen and FN. The beneficial effects of clopidogrel on the renal deposition of collagen in the kidneys of *db/db* mice may be explained by its inhibition of diabetes-associated macrophage infiltration and proinflammatory cytokine release. However, further research is required to determine whether there is a genuine cause-and-effect link between these findings. In addition, the lower macrophage infiltration may explain the reduction in ECM accumulation and fibrosis. However, it may be that clopidogrel also directly inhibits renal inflammation and fibrosis. This possibility requires further investigation.



DOI: 10.4239/wjd.v13.i8.600

Figure 4 Clopidogrel inhibits the expression of tumor necrosis factor-α and interleukin-1β in *db/db* mice. Data are shown as mean ± SD values of $n = 6$ per group. [a]$P < 0.05$ *vs* wild-type mice; [b]$P < 0.05$ *vs* *db/db* mice. A: Tumor necrosis factor (TNF)-α and interleukin (IL)-1β gene expression, determined using real-time polymerase chain reaction; B: Immunohistochemical staining; C: Semi-quantitative analysis of TNF-α and IL-1β protein expression. WT: Wild type; *db/db*: *db/db* mice; Clo: Clopidogrel; TNF: Tumor necrosis factor; IL: Interleukin.

CONCLUSION

We found that clopidogrel significantly reduced renal collagen deposition and fibrosis and prevented renal dysfunction in *db/db* mice. These findings suggest a promising alternative approach to the treatment of diabetes and prevention of DN because clopidogrel is in current use and could be co-administered with other antidiabetic drugs.

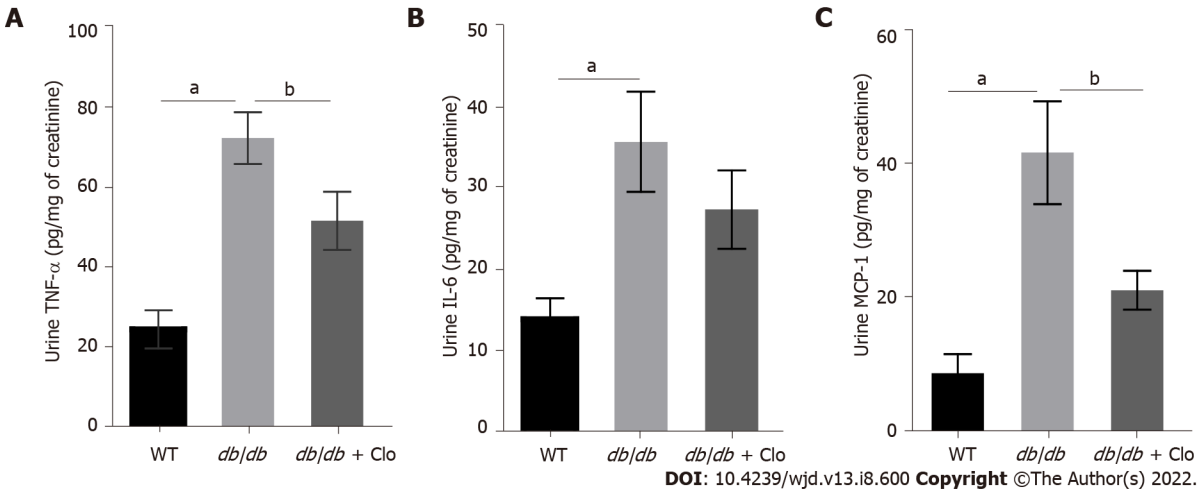


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Figure 5 Clopidogrel reduces the levels of urinary tumor necrosis factor-α and monocyte chemoattractant protein-1 in *db*/*db* mice. Data are shown as mean ± SD values of $n = 6$ mice per group. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. A: Tumor necrosis factor-α levels in the urine; B: Interleukin-6 levels in the urine; C: Monocyte chemoattractant protein-1 levels in the urine. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel 20 mg/kg; TNF: Tumor necrosis factor; IL: Interleukin; MCP: Monocyte chemoattractant protein.

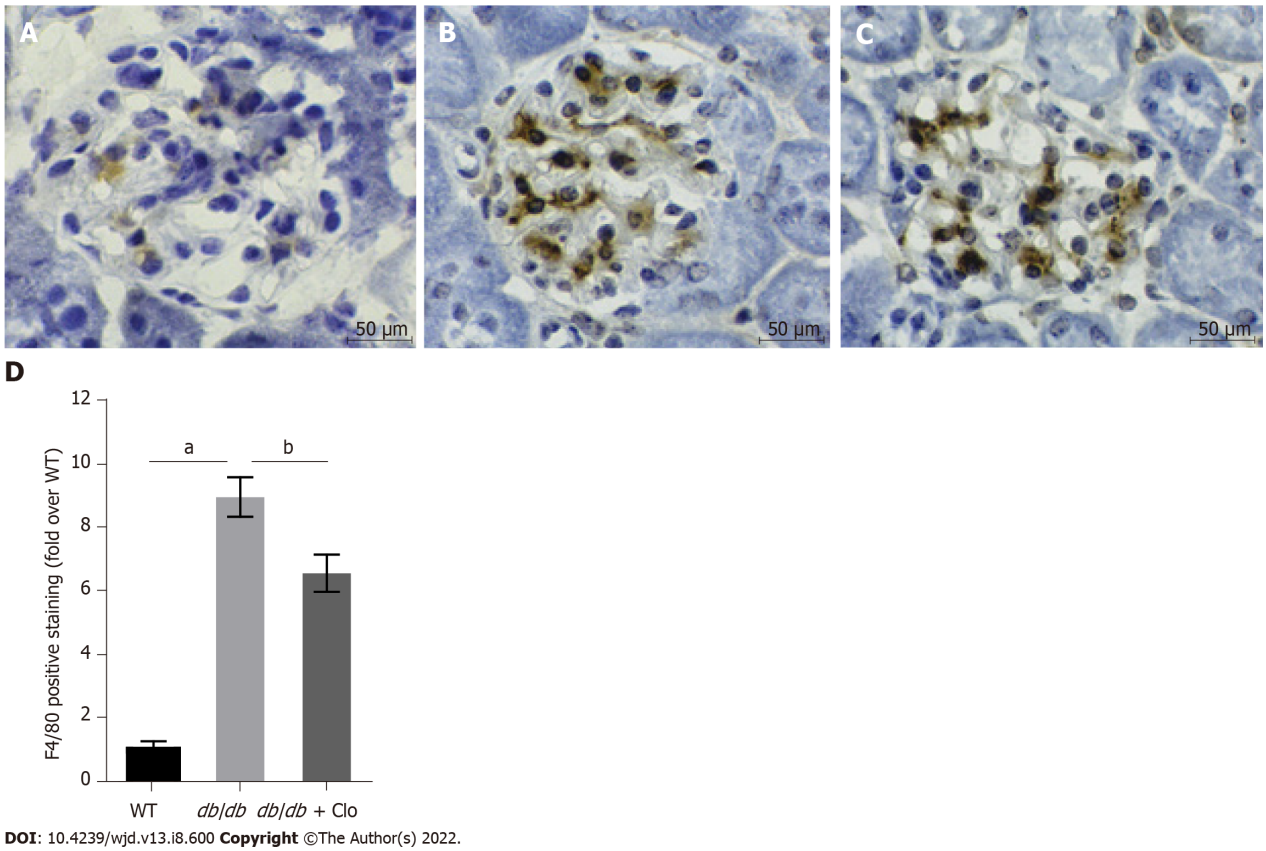

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Figure 6 Clopidogrel reduces renal macrophage infiltration in *db*/*db* mice. [a]$P < 0.05$ *vs* wild type mice; [b]$P < 0.05$ *vs* *db*/*db* mice. Immunohistochemical staining, magnification: × 400. A: Wild type; B: *db/db* mice; C: *db/db* + clopidogrel (20 mg/kg); D: Immunohistochemical analysis of F4/80 expression. WT: Wild type; *db/db*: *db*/*db* mice; Clo: Clopidogrel.

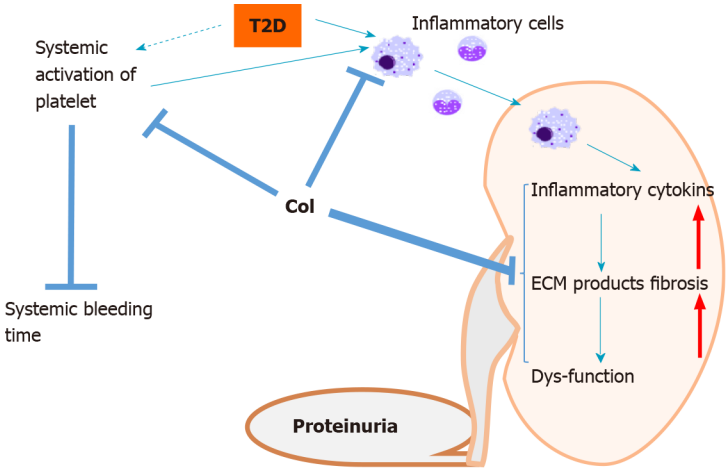

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Figure 7 Outline of the potential mechanisms whereby clopidogrel ameliorates defects in renal structure and function in *db*/*db* mice. Diabetes is associated with the activation of platelets, predisposing toward systemic thrombosis, which is reflected in a shorter bleeding time. Diabetes is also characterized by the activation of inflammatory cells, including macrophages, which infiltrate the kidney and release proinflammatory cytokines, causing systemic and renal inflammation, which is followed by renal damage, remodeling, and dysfunction. The treatment of *db*/*db* mice with clopidogrel may exert its effects in three main ways: (1) Direct inhibition of the infiltration of circulating inflammatory cells into the kidney; (2) Inactivation of activated platelets, preventing their promotion of inflammatory cell infiltration; and (3) Direct prevention of the diabetes-related renal inflammatory response and associated downstream pathways. The dashed arrow indicates that the systemic activation of platelet aggregation in diabetes requires further confirmation in longer-term studies of diabetes models. T2D: Type 2 diabetes; ECM: Extracellular matrix.

ARTICLE HIGHLIGHTS

Research background

Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease in the United States and most developed countries. New strategies are required to delay the development and the progression of DN.

Research motivation

Previous studies have shown that clopidogrel administration is an effective means of suppressing inflammation in diabetes. Moreover, clopidogrel can ameliorate diabetes-induced renal fibrosis in a streptozotocin-induced murine model of type 1 diabetes.

Research objectives

We aimed to determine whether treatment with clopidogrel has a preventive or therapeutic effect in the kidneys of obese type 2 diabetic *db/db* mice.

Research methods

Clopidogrel at doses of 5, 10, or 20 mg/kg was administered by gavage for 12 wk. The body mass, blood glucose, and urinary creatinine and albumin concentrations were measured. Immunohistochemistry, enzyme-linked immunosorbent assay and real-time quantitative polymerase chain reaction were used to evaluate the expression of cytokines. Fibronectin (FN), and collagen I was assessed using immunohistochemistry.

Research results

Clopidogrel treatment reduced urinary albumin/creatinine ratio. Immunohistochemical staining revealed an amelioration of renal fibrosis, significantly less deposition of FN and collagen I. Lower expression of the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β and lower levels of urinary TNF-α, monocyte chemoattractant protein-1 and significantly reduced macrophage infiltration of the *db/db* mice.

Research conclusions

Clopidogrel prevented renal dysfunction in *db/db* mice, most likely through inhibition of renal macrophage infiltration and the associated inflammation.

Research perspectives

The present findings suggest a promising alternative approach to the treatment of patients with diabetes

and the prevention of DN because clopidogrel is in current use and could be co-administered with other antidiabetic drugs.

FOOTNOTES

Author contributions: Pei J and Li HQ contributed to conception and design of the study; Li HQ and Liu N performed the experiment; Zheng ZY organized the database; Teng HL performed the statistical analysis; Li HQ and Liu N wrote the draft of the manuscript; and all authors contributed to manuscript revision, read, and approved the submitted version.

Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Diabetes

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World J Diabetes 2022 August 15; 13(8): 613-621

DOI: 10.4239/wjd.v13.i8.613

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Improved systemic half-life of glucagon-like peptide-1-loaded carbonate apatite nanoparticles in rats

Nabilah Ibnat, Rahela Zaman, Mohammad Borhan Uddin, Ezharul Chowdhury, Chooi Yeng Lee

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Arslan M, Turkey; Dziegielewska-Gesiak S, Poland

Received: December 15, 2021
Peer-review started: December 15, 2021
First decision: January 12, 2022
Revised: January 24, 2022
Accepted: July 16, 2022
Article in press: July 16, 2022
Published online: August 15, 2022

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Abstract

BACKGROUND

Glucagon-like peptide-1 (GLP1) is an endogenous peptide that regulates blood glucose level. But its susceptibility to rapid metabolic degradation limits its therapeutic use.

AIM

To prepare GLP1-encapsulated nanosize particle with controlled release property to improve the systemic half-life of GLP1.

METHODS

GLP1 nanoparticles were prepared by complexation of GLP1 with carbonate apatite nanoparticles (CA NPs). The physicochemical properties of the CA NPs, the effects of GLP1-loaded CA NPs on cell viability, and the systemic bioavailability of GLP1 after CA NPs administration were determined.

RESULTS

The GLP1-loaded CA NPs was within 200 nm in size and stable in fetal bovine serum. The formulation did not affect the viability of human cell lines suggesting that the accumulation of CA NPs in target tissues is safe. In Sprague Dawley rats, the plasma GLP1 Levels as measured from the GLP1-loaded CA NPs-treated rats, were significantly higher than that of the control rats and free GLP1-treated rats at 1 h post-treatment ($P < 0.05$), and the level remained higher than the other two groups for at least 4 h.

CONCLUSION

The GLP1-loaded CA NPs improved the plasma half-life of GLP1. The systemic bioavailability of GLP1 is longer than other GLP1 nanoparticles reported to date.

Key Words: Glucagon-like peptide-1; Metabolic syndrome; Nanoparticles; Plasma half-life; Rat

Core Tip: Glucagon-like peptide-1 (GLP1), owing to its physiological properties, is a promising peptide in the treatment of obesity and diabetes. Due to the short half-life of GLP1 and in order to improve GLP1 therapeutic use, GLP1 receptor agonists (GLP1-RAs) have been widely synthesised and encapsulated into nanocarriers for targeted delivery. But the use of GLP1-RAs is associated with unwanted side effects and risks. In the present study, we synthesised a new nanocarrier for native GLP1 - the GLP1 carbonate apatite nanoparticles. The nanocarrier appears comparable if not significantly better than other GLP1 nanoparticles, which have shown promising features as therapeutic agents.

Citation: Ibnat N, Zaman R, Uddin MB, Chowdhury E, Lee CY. Improved systemic half-life of glucagon-like peptide-1-loaded carbonate apatite nanoparticles in rats. *World J Diabetes* 2022; 13(8): 613-621
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/613.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.613

INTRODUCTION

The incretin hormone glucagon-like peptide-1 (GLP1) is a peptide secreted from the L cells of the distal ileum and colon in response to nutrient to stimulate insulin secretion. However, as with other peptides, GLP1 is rapidly broken down by circulating enzyme dipeptidyl peptidase-IV (DPP-IV). The poor bioavailability of peptide-based therapeutics is the main challenge of achieving maximum benefit from the drugs.

The metabolic instability of GLP1 has led to the development of GLP1 receptor agonists (GLP1-RAs), which are now widely used in diabetic treatments. GLP1-RAs are generally delivered as payload of drug carriers in injectable formulation. Besides retaining the physiological functions of GLP1, GLP1-RAs have shown additional effects such as regulation of body weight, blood pressure, and cholesterol level [1,2]. But the use of GLP1-RAs was accompanied by side effects such as nausea, vomiting, adverse injection-site reaction, and has posed the risks for pancreatitis and thyroid cell carcinomas[2]. Another aspect of the GLP1-RAs preparations that is worth considering is their pharmacokinetics profile, which essentially is determined by the properties of the drug carriers. Systemic bioavailability of the same drug varies depending on the route of administration that the drug carriers are designed for.

Frequent parenteral administration of therapeutic peptides may lead to a lower patient compliance as compared to oral administration, and also increase the chance of side effects. Moreover, systemically administered peptide drugs have very short half-lives owing to renal clearance and their interaction with the host immune system[3], thus necessitating multiple administration over the course of treatment, which may in turn causes systemic toxicity and undesirable off-target effects. The drawback of GLP1 has also led to the common strategies of conjugating GLP1 or its analogues to polyethylene-glycol (PEG)[4,5] and albumin[6,7] with the aim to extend the peptide's half-life. However, the efficiency and long-term safety of these conjugates have limited their use[6]. Of note, most of these chemical conjugations was done on GLP1-RAs.

Controlled release formulation is a promising approach as it releases the peptide molecule depending on the needs. Such formulation is therefore able to maintain the circulating level of the peptide and prolong therapeutic activities[8-10]. Previously, we demonstrated that pH sensitive inorganic carbonate apatite nanoparticles (CA NPs) were excellent carriers for intracellular delivery of deoxyribonucleic acid (DNA). We have reported the properties of the CA NPs including their sizes, distribution and zeta potential, measurements from the fourier transform-infrared spectroscopy (FT-IR) and x-ray diffraction and dissolution studies, and effects on crystal growth kinetics[11].

In this study, GLP1-loaded CA NPs was formulated, and the *in vitro* and *in vivo* properties of the NPs, specifically their ability to improve the systemic half-life of GLP1 were assessed. We asked the following questions: (1) Does GLP1-CA NPs increase the systemic bioavailability of GLP1 as compared to free GLP1? And (2) Will GLP1-CA NPs with controlled release properties improve the systemic half-life of GLP1 to a similar extent to that of GLP1-RAs?

MATERIALS AND METHODS

Fabrication of GLP-1-loaded CA NPs

The CA NPs was prepared by dissolving 44 mmol/L of sodium bicarbonate and Dulbecco's Modified Eagle Medium (DMEM) powder in mili Q water (pH adjusted to 7.4). The DMEM solution was then mixed with 7 mmol/L concentration of calcium chloride ($CaCl_2$), followed by 30 min incubation at 37°C. For the complexation of GLP1 with CA NPs, a series of GLP1 concentrations ranging from 10 µg to 2 mg, was added to the DMEM solution prior to the addition of 7 mmol/L $CaCl_2$, and the preparations were incubated at 37°C for 30 min.

Turbidity measurement of GLP1-loaded CA NPs

Turbidity measurement was carried out to determine the growth of the particles. The CA NPs and the GLP1-loaded CA NPs were formulated, as described above. The turbidity of the particle suspensions was assessed using ultraviolet (UV) spectrophotometer at 320 nm absorbance wavelength (UV 1800 Spectrophotometer, Shimadzu, Japan).

Visualisation of GLP1-loaded CA NPs under a Field Emission-Scanning Electron Microscope

The field emission-scanning electron microscope (FE-SEM) was used to observe the morphology of the NPs. The GLP1-CA NPs samples were prepared by adding GLP1 (1 and 10 µg) along with 4 mmol/L $CaCl_2$ to 1 mL bicarbonate-buffered medium containing inorganic phosphate. One drop of the complex particle suspension was dried on a glass slide at 37°C for 1 h. The slide was placed onto a carbon tape-coated sample holder. The dried samples underwent platinum sputtering with 30 mA sputter current at 2.30 tooling factor for 70 s, and the sputtered particles visualised at 5.00 kV (Hitachi/SU8010, Tokyo, Japan).

Binding affinity of GLP1 to CA NPs

Fetal bovine serum (FBS, 1%) was used as a source of serum protein to test the binding affinity of GLP1 to CA NPs. The CA NPs containing 5 mmol/L $CaCl_2$ was prepared under the same condition as mentioned above. The CA NPs was then added with 1% FBS and incubated for 10 min. The NPs, coupled with different concentrations of GLP1 - 500 µg, 1 mg and 2 mg were incubated for 30 min at 37°C. These samples together with the free GLP1 were centrifuged at 13000 rpm for 10 min, where the supernatant was discarded. The pellet was washed with 1 mL DMEM before being dissolved with ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (50 mmol/L). The samples were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) in 1% agarose gel. The gel was fixed in a fixing solution for 1 h, stained with Coomassie Blue for 20 min with gentle agitation, and de-stained in a de-staining solution. The image of the de-stained gel was captured using the gel documentation system from Bio-Rad (The United States of America, United States).

Effect of GLP1-CA NPs on the viability of human cell line

The human Michigan Cancer Foundation-7 (MCF-7) cell line, which is a breast cancer cell line, was grown in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin antibiotic in a 25 mm^3 culture flask. One day before the treatment, the exponentially growing cells were trypsinised, centrifuged and re-suspended using DMEM. Cells were counted under the optical microscope using a haemocytometer and seeded on a 24-well plate with cell density of 50000 cells per well. The cells were allowed to attach overnight at 37°C with 5% carbon dioxide (CO_2). Cells were then treated with either free GLP1 or GLP1-CA NPs in the presence or absence of 10% FBS, and for different length of time prior to the cytotoxicity study using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT). In the MTT assay, 50 µL MTT (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 4 h. The formazan products were dissolved with 300 µL dimethyl sulfoxide, and the absorbance measured at 595 nm wavelength with reference to 630 nm on a microplate reader (Dynex Opsys MR, United States).

Measurement of plasma GLP1 Levels following intravenous injection in rats

The experiment was approved by the Monash University Animal Ethics Committee (MARP/2016/008). Male Sprague Dawley rats (n = 18, 6 wk old) were obtained from the Monash Animal Facility and handled according to the appropriate animal care guidelines. Each rat was housed in an individually ventilated cage, in a temperature- and humidity-controlled room with a 12 h light-dark cycle (lights on 06:00-18:00), and was allowed free access to control diet (Gold Coin Sdn. Bhd.) and water. After 7 d of acclimatisation period, the rats were divided into three groups (n = 6 per group), and administered *via* the tail vein, one of the following preparations: CA NPs only, 1 mg/kg free GLP1, and GLP1-loaded CA NPs containing 1 mg/kg GLP1. Approximately 300 µL of blood sample was collected from the tail vein and transferred to a sterile 1.5 mL EDTA-coated tube. Samples were collected at 0 h (pre-treatment), and 1, 2, 4, and 24 h post-treatment, and the blood was centrifuged at 3000 rpm for 15 min at 4°C to separate out the plasma, which was then stored at -20°C. The plasma levels of GLP1 were measured using a commercially available GLP1 enzyme-linked immunosorbent assay (ELISA) kit (Millipore, United

States).

Statistical analysis

Results were presented as mean ± standard error of the mean (SEM). The statistical significance of the treatment groups compared to the control was analysed using the Student's *t* test. Student's *t* test is a well-established parametric statistical method that compares the means of two independent groups and is more appropriate to be used when sample size is small. A *P* value of less than 0.05 was considered statistically significant[12].

RESULTS

The growth profile of GLP1 nanoparticles

As illustrated in Figure 1, increasing the concentration of free GLP1 in the DMEM solution did not change the absorbance intensity of GLP1. An inverse relationship between GLP1 concentrations (ranged from 10 μg to 2 mg) and the formation of CA NPs was found. This indicates that GLP1 might interact with the growing CA NPs and modulate the NPs growth kinetics.

Size and elemental analysis

As shown in Figure 2A, CA NPs was approximate 200 nm in sizes. The presence of GLP1 (1 and 10 μg) along with 4 mmol/L $CaCl_2$ yielded particles of heterogeneous sizes. Accordingly, 1 μg GLP1 gave rise to particle sizes ranging from approximate 15 to 200 nm (Figure 2B), while 10 μg GLP1 produced particles with intermediate sizes ranging from approximate 60 to 70 nm (Figure 2C). Energy Dispersive X-Ray Analysis-based elemental analysis showed that particles formed with 10 μg GLP1 contained more carbon than particles formed with 1 μg GLP1, which may be explained by the presence of higher amount of hydrocarbon in the particles formed with 10 μg GLP1. In addition, the calcium/phosphate (Ca^{2+}/P) ratio was found to be much higher in particles fabricated with higher amount of GLP1, probably as a result of phase transformation (data not shown).

Binding affinity of GLP1 towards CA NPs

In SDS-PAGE, a GLP1 band along with a FBS band was observed for CA NPs prepared with 1 mg and 2 mg GLP1. A more prominent GLP1 band was seen from the latter (Figure 3). Results confirmed that there was sufficiently stable complex formation between the NPs and GLP1. Serum proteins did not trigger the dissociation of GLP1 from the CA NPs.

Effect of GLP1-CA NPs on the viability of human cell line

In the MTT assay, both the free GLP1 and CA NPs-bound GLP1 did not exert noticeable effects on the viability of the human cell line (Figure 4). This suggests that the GLP1-loaded CA NPs are likely to be safe for clinical application. More pre-clinical studies are needed to confirm this.

Plasma half-life of GLP1

The plasma levels of GLP1 in rats treated with GLP1-CA NPs were significantly higher than control rats at 1 h (49.61 ± 9.24 picomolar (pM), $P < 0.05$), 2 h (20.22 ± 5.20 pM, $P < 0.05$), and 4 h (16.32 ± 4.01 pM, $P < 0.05$) (Figure 5). The increased plasma GLP1 Level at 1 h in GLP1-CA NPs treated rats was also significantly higher than the levels measured from rats administered with free GLP1 (15.68 ± 4.34 pM, $P < 0.05$).

DISCUSSION

Therapeutic drug with a high tendency of reaching its target site may have increased efficiency and limited side effects. Nanosize particle has the advantage in this aspect as it allows targeted drug delivery. In the present study, a new GLP1-loaded nanosize particle with controlled release property was successfully developed. The formulation adds to the list of GLP1 nanoparticles reported to date, which has made little progress since our review of clinically available GLP1 NPs for diabetic treatment 5 years ago[13].

The slow progress or lack of interest in developing particles that encapsulate native GLP1 could be attributed to the rapid metabolism of GLP1, and as such, making GLP1-RAs a potentially more viable option. The only NPs preparation for intravenous GLP1 administration, a study design closest to the present study, was reported more than a decade ago[14]. The preparation, which used liposome as the drug carrier, produced a 3.6-fold higher serum GLP1 Level than that of free GLP1 at 15 min post-treatment. But the elevated GLP1 Level decreased rapidly thereafter. Another preparation, which was comparable to the present study in terms of the administrative dose and test subject, and involved a

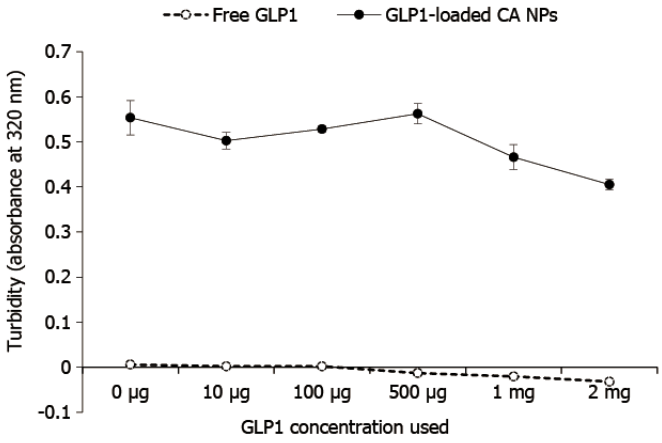

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Figure 1 Turbidity measurement. The turbidity of samples measured at absorbance 320 nm was an indicator of carbonate apatite nanoparticles (CA NPs) particles growth. The growth of glucagon-like peptide-1 (GLP1)-loaded CA NPs declined in inverse proportion as the concentration of GLP1 increased.

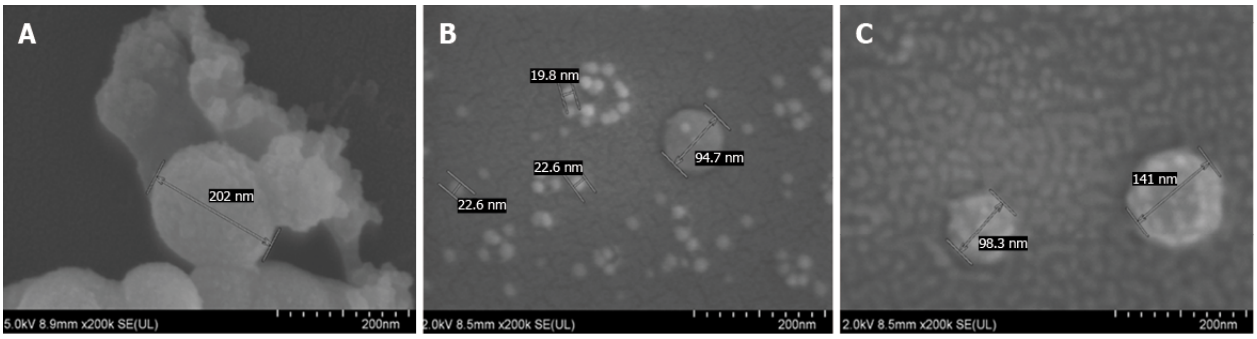

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Figure 2 The field emission-scanning electron microscope images of the carbonate apatite nanoparticles. Particles approximate 200 nm in size were formed when carbonate apatite nanoparticles (CA NPs) were fabricated without glucagon-like peptide-1 (GLP1) (A). When the CA NPs were fabricated with 1 μg GLP1 (B) and 10 μg GLP1 (C), various sizes of particles were observed with the latter preparation gave a greater size heterogeneity.

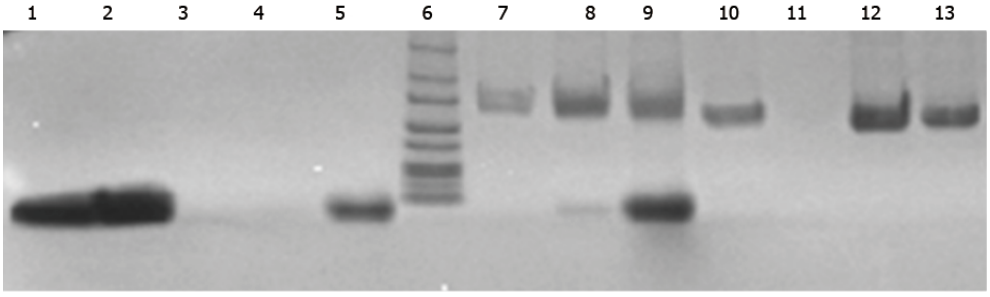

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Figure 3 The binding affinity of glucagon-like peptide-1 towards carbonate apatite nanoparticles. Lane 1. Free glucagon-like peptide-1 (GLP1) 50 μg; 2. Free GLP1 100 μg; 3. GLP1 500 μg + Ca^{2+}; 4. GLP1 1 mg + Ca^{2+}; 5. GLP1 2 mg + Ca^{2+}; 6. protein ladder; 7. GLP1 500 μg + Ca^{2+} +1% fetal bovine serum (FBS); 8. GLP1 1 mg + Ca^{2+} + 1% FBS; 9. GLP1 2 mg + Ca^{2+} + 1% FBS; 10. Dulbecco's Modified Eagle Medium (DMEM) + Ca^{2+} + 1% FBS; 11. Blank; 12. 1% FBS; 13. Ca^{2+} + 1% FBS. GLP1 band was observed from carbonate apatite nanoparticles prepared with 1 mg and 2 mg GLP1, as shown in Lanes 8 and 9, along with the FBS band.

silica-based pH sensitive nanomatrix system, showed a burst release of GLP1 during the first hour. The plasma level of GLP1 however, returned to basal level at 4 h[15].

The GLP1-CA NPs have improved the systemic bioavailability of GLP1, showing better sustained release properties than the liposomal carrier and pH sensitive nanomatrix system. The plasma GLP1 Level of the CA NPs-treated rats was 3.2-fold higher compared to the free GLP1-treated rats at 1 h, and

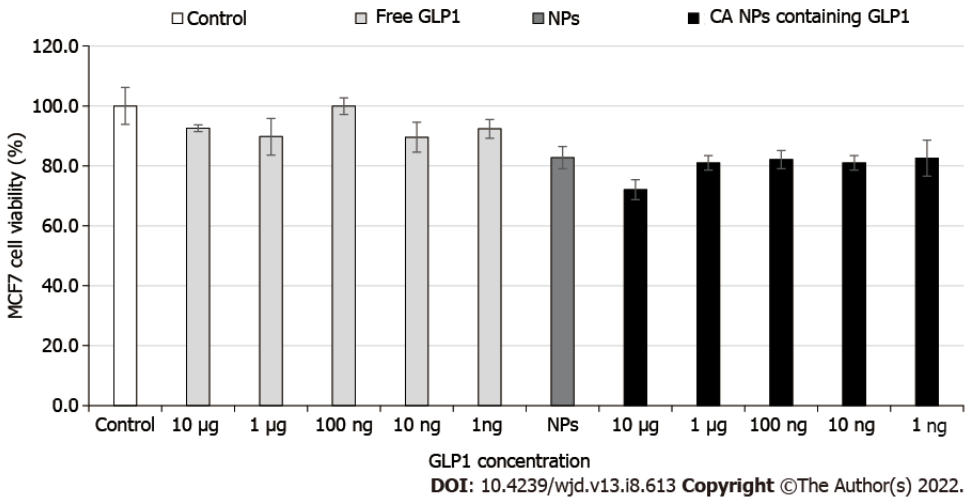

Figure 4 The effect of glucagon-like peptide-1 nanoparticles on the viability of Michigan Cancer Foundation-7 cells. The carbonate apatite nanoparticles with increasing glucagon-like peptide-1 concentrations did not affect the viability of the cells.

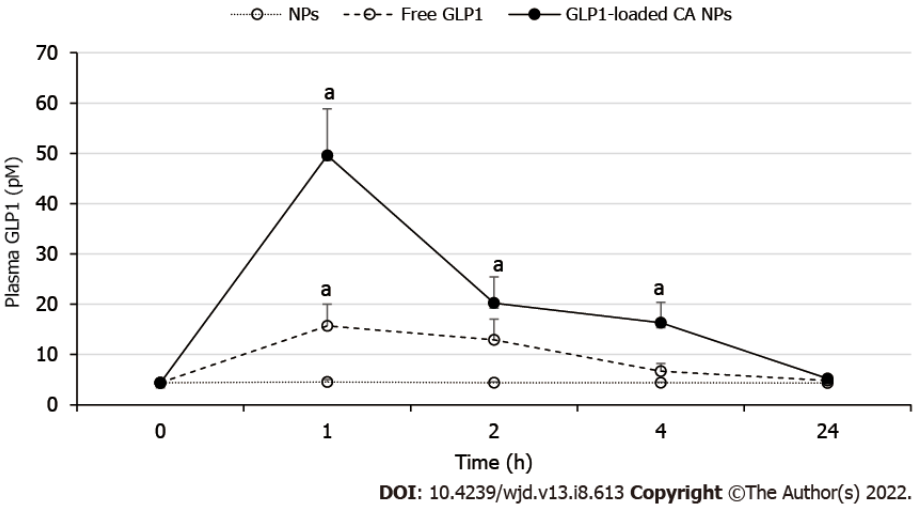

Figure 5 Plasma-time profile of glucagon-like peptide-1. At 1 h post-treatment, Sprague Dawley rats (n = 6) administered with carbonate apatite nanoparticles containing 1 mg/kg of glucagon-like peptide-1 (GLP1) showed significantly higher plasma GLP1 level than the control rats and 1 mg/kg of free GLP1-treated rats ($^aP < 0.05$). The increased plasma GLP1, which was continued to be seen at 4 h, showed that GLP1 was released from the particles in a controlled manner.

the level was sustained for at least 4 h post-treatment. Although carbonate apatite was reported to have strong affinity toward bovine serum albumin at physiological pH[16], the observed high binding affinity of GLP1 towards CA NPs may have negated the potential interaction between CA NPs and various blood proteins.

The liposomal preparation[14], and the silica-based pH sensitive nanomatrix system[15] mentioned above reported significant reduction in the glucose level, and the liposomal GLP1 also significantly increased insulin secretion. Given that CA NPs showed superior plasma GLP1 profile to these preparations, it is reasonable to predict that GLP1-loaded CA NPs will exert similar insulinotropic and hypoglycaemic effects. Nonetheless, further studies are necessary to confirm the therapeutic effects of GLP1-CA NPs.

Despite having a short plasma half-life, GLP1 may still be preferred to GLP1 agonists for therapeutic use. This is evidenced in several studies which used nanomaterials without GLP1 or GLP1-RAs as payload to stimulate GLP1 secretion[5,17,18]. The surface-modified lipid-based nanocarriers not only increased GLP1 secretion significantly, but normalised plasma glucose levels, and reduced insulin resistance in obese/diabetic mice following a 4 wk treatment[5].

Liraglutide, exenatide and exendin-4 are GLP1-RAs widely used to improve the therapeutic effects of GLP1. It is therefore useful to understand the pharmacokinetic/pharmacodynamic profiles of GLP1-RA-loaded carrier systems so as to gain a better understanding of how significant these preparations had in

improving GLP1 bioavailability when comparing with GLP1 nanoparticles, and this was reviewed recently[19]. In order to make comparison between GLP1-CA NPs and GLP1-RAs preparations for their systemic bioavailability and usefulness, only studies that used rats as test subjects and preparations meant for oral administration were discussed below.

Exendin-4 released from enteric-coated capsule containing pH responsive NPs showed a maximum plasma concentration at 5 h after treatment[20]. Another preparation that involved poly(lactic-co-glycolic acid) NPs conjugated with dextran and a C-terminal Src kinase peptide showed peak plasma exenatide level at 6 h post-administration. However, the elevated plasma exenatide level was not significantly different from rats receiving subcutaneous injection of exenatide solution, which was used as a positive control[21]. Zhang *et al*[22,23] who used functionalised NPs for oral exenatide delivery, reported maximum plasma exenatide level at 4 h and 6 h, respectively after administration. But similar to the study by Song *et al*[21], the exenatide level was not significantly different from that seen in rats administered subcutaneously with an exenatide solution. Overall, the sustainability of the plasma concentration of GLP1-RAs encapsulated in various types of nanomaterials was between 4-6 h, only slightly higher than that of GLP1-encapsulated CA NPs, although an oral formulation may produce better patient compliance than parenteral administration.

On this note, it is necessary to highlight that the GLP1 systemic bioavailability and/or the biological effects of the different nanomaterial preparations discussed above were cited from and compared between animal subjects. Preparations which are under pre-clinical testing stages, as per the present study, provide useful information on the potential usability and practicality of the preparations in human, and are instructive for future investigation on other animal species and subsequently, human subjects.

Liraglutide and semaglutide have been approved by the United States Food and Drug Administration as weight management agents, and semaglutide in obese or overweight adults with at least one weight-related condition including diabetes. Emerging evidences support the implementation of pharmacotherapy along with behavioural therapy and dietary intervention in optimising obesity treatment. The same approach may apply to diabetes management because consumption of food with antioxidant and anti-inflammatory properties could synergise the effects of GLP1 and GLP1-RAs[24]. If combination therapy is the recommendation for the treatment of metabolic syndrome, the overall cost of management should be taken into consideration.

This study was limited by the lack of chronic and sub-chronic *in vivo* study involving obese or diabetic animals. A longer duration of study and measurement of metabolic parameters will provide evidence of the effectiveness of GLP1 CA NPs in the long-term treatment of metabolic syndrome. Nevertheless, we showed that this new preparation has therapeutic potential. A second limitation is on the discussion of the data itself. In order to make valid and comparable comparison of the pharmacokinetics profiles between the different GLP1 nanocarriers, only studies that used the same animal species, and native GLP1 were included in the discussion. This means that not all GLP1 and GLP1 RAs nanocarriers that are reported to date have been included. The literature search has nevertheless implied that there is higher interest in GLP1-RAs than native GLP1 despite the high cost of producing GLP1-RAs.

CONCLUSION

In summary, we have developed a new nanocarrier for GLP1. *In vitro*, the GLP1-CA NPs are safe on human cell lines, and stable against dissociation from interaction with plasma proteins. *In vivo*, GLP1-CA NPs demonstrated sustained release properties by significantly improving the plasma half-life of GLP1. The preparation has resulted in a better GLP1 systemic bioavailability than previously reported GLP1-loaded nanocarriers, making it a potentially promising treatment option for metabolic syndrome.

ARTICLE HIGHLIGHTS

Research background

Apart from Glucagon-like peptide-1 (GLP1) receptor agonists that are being widely used and studied, more effort should also be channeled to designing carrier with sustained release properties for native GLP1 because both approaches may be equally effective in improving the systemic half-life of GLP1.

Research motivation

The GLP1-carbonate apatite nanoparticles (CA NPs) overcome the short half-life of GLP1. The nanoparticles could be a potential therapeutic option for metabolic syndrome and warrant further investigation.

Research results

A stable GLP1-CA NPs was successfully fabricated. The NPs improved the systemic half-life of GLP1 as compared with free GLP1-treated rats. The increased plasma GLP1 Level was maintained for at least 4 h post-treatment.

Research methods

The nanoparticles were fabricated through complexation between GLP1 and CA NPs. The GLP1-CA NPs was then evaluated for physicochemical properties, tested for their potential cytotoxic effects on human cell line, and finally measured for systemic bioavailability in rats through intravenous administration.

Research objectives

To fabricate GLP1-loaded carbonate apatite nanoparticles (GLP1-CA NPs), and improve the systemic half-life of GLP1 through GLP1-CA NPs.

Research conclusions

pH sensitive inorganic carbonate apatite nanoparticles, which we have successfully formulated previously may be a potential carrier for GLP1.

Research perspectives

GLP1 is an endogenous peptide with established glucose lowering property. Its therapeutic use however is limited due to it being rapidly degraded in the systemic circulation. Nanosize particles with sustained release property may protect as well as extend the plasma half-life of GLP1.

ACKNOWLEDGEMENTS

The authors would also like to thank Md. Karim E for his assistance in the *in vivo* study.

FOOTNOTES

Author contributions: Lee CY and Chowdhury E contributed to the study concept and design; Ibnat N, Zaman R and Uddin B acquired the data; Ibnat N, Uddin B, Lee CY and Chowdhury E analysed the data; Ibnat N drafted the manuscript; Lee CY reviewed and edited the manuscript; all authors have read and approved the final manuscript.

Supported by an FRGS grant from the Ministry of Education, Malaysia, No. FRGS/2/2014/SG05/MUSM/03/1.

Institutional animal care and use committee statement: The study was reviewed and approved by the Monash University Animal Ethics Committee (Approval No. MARP/2016/008).

Conflict-of-interest statement: There is no conflict-of-interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: Malaysia

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S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 622-642

DOI: 10.4239/wjd.v13.i8.622

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

In vivo evaluation and mechanism prediction of anti-diabetic foot ulcer based on component analysis of Ruyi Jinhuang powder

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bahrmann A, Germany; Elnakib AA, Egypt; Lee AS, Australia

Received: May 6, 2022
Peer-review started: May 6, 2022
First decision: May 30, 2022
Revised: June 10, 2022
Accepted: July 6, 2022
Article in press: July 6, 2022
Published online: August 15, 2022

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Abstract

BACKGROUND

Diabetes is a metabolic disease with a high complication rate. Diabetic foot ulcers (DFUs) seriously affect the quality of life of patients. A total of 15%-20% of diabetic patients develop DFUs, which heal with difficulty over a long time and can result in amputation and disability. Traditional Chinese medicine has a unique effect in the treatment of skin ulcerative diseases. Ruyi Jinhuang powder (RHP) is one of the classic prescriptions in traditional Chinese medicine and is widely used in clinical practice.

AIM

To verify the ability of RHP to promote wound healing by electron microscopy analysis in animal models and hematoxylin-eosin (HE) staining. The effective components of RHP were extracted and identified by gas chromatography-mass spectrometry (GC-MS), and the obtained chemical components were analyzed by network pharmacology methods to predict its therapeutic mechanism.

METHODS

Sprague Dawley rats were injected with streptozotocin to establish the DFU model. HE staining was used to observe the wound tissue under an electron microscope. The chemical constituents of RHP were extracted first by supercritical fluid extraction and alcohol extraction, and then, GC-MS and ultra-performance

liquid chromatography–MS were used to separately identify the chemical constituents. In addition, the "herb-component-target" link was established through the Traditional Chinese Medicine Systems Pharmacology database to obtain the target information, and the molecular docking of important components and key targets was performed in Discovery Studio software. Cytoscape software was used to visualize and analyze the relationship between the chemical composition, targets and Traditional Chinese Medicine network.

RESULTS

RHP promoted DFU healing in rats by affecting fibroblasts and nerve cells. A total of 89 chemical components were obtained by GC-MS. Network pharmacological analysis revealed that RHP was associated with 36 targets and 27 pathways in the treatment of DFU, of which the important components were luteolin, trans caryophyllene, ar-turmerone, palmitic acid, methyl palmitate, gallic acid, demethoxycurcumin, berberine, and rheic acid. The key targets were posttranscriptional silencing, topoisomerase II alpha, muscarinic acetylcholine receptor M2, interleukin 6, tumor necrosis factor and retinoic X receptor alpha, and the key pathways were the phosphoinositide 3-kinase-protein kinase B signaling pathway, neuroactive ligand–receptor interactions, and the forkhead box O signaling pathway.

CONCLUSION

Our results indicated that RHP may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses and regulating cell proliferation, differentiation and apoptosis through other specific mechanisms.

Key Words: Ruyi Jinhuang powder; Diabetic foot ulcer; Mass spectrometry-chromatography; Network pharmacology; Hematoxylin-eosin staining; Components analysis

Core Tip: Although some studies have suggested that Ruyi Jinhuang powder (RHP) has a therapeutic effect on diabetic foot ulcers (DFUs), few have used component analysis, investigated the mechanism of action, and utilized wound-healing experiments. The components of RHP were used to predict the mechanism of action, and wound healing was observed by establishing a DFU rat model to further prove the therapeutic effect of RHP on DFU to finally determine a possible mechanism of action.

Citation: Li XY, Zhang XT, Jiao YC, Chi H, Xiong TT, Zhang WJ, Li MN, Wang YH. *In vivo* evaluation and mechanism prediction of anti-diabetic foot ulcer based on component analysis of Ruyi Jinhuang powder. *World J Diabetes* 2022; 13(8): 622-642

URL: https://www.wjgnet.com/1948-9358/full/v13/i8/622.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i8.622

INTRODUCTION

Ruyi Jinhuang powder (RHP) is included in "Authenticity of Surgery" written by Chen Shigong in the Ming Dynasty, which includes "*THF* [*Trichosanthin* (*Tian Hua Fen*)], *DH* [*Rhubarb* (*Da Huang*)], *HB* [*Phellodendron* (*Huang Bai*)], *JH* [*Turmeric* (*Jiang Huang*)], *BZ* [*Angelica dahurica* (*Bai Zhi*)], *TNX* [*Arisaema* (*Tian Nan Xing*)], *CZ* [*Atractylodes lancea* (*Cang Zhu*)], *HP* [*Magnolia officinalis* (*Hou Po*)], *CP* [*Pericarpium Citri Reticulatae* (*Chen Pi*)] and *GC* [*Glycyrrhiza uralensis* (*Gan Cao*)]"[1] and is now included in the Chinese Pharmacopoeia. This prescription is used for swelling relief and pain relief. In this prescription, *Trichosanthes* is the monarch medicine, and the minister medicine *rhubarb* with the same cold and bitter taste is used to purge fire detumescence; *angelica dahurica* and *turmeric* are the minister pungent medicines with compatibility to discharge pus pain; *pericarpium citri reticulatae*, *Magnolia officinalis*, *atractylodes lancea and glycyrrhiza uralensis* are combined to remove dampness and regulate Qi; and *arisaema* alleviates swelling pain. The above five medicines act together as adjuvants with *glycyrrhiza uralensis* to reconcile and detoxify the medicine. Modern clinical applications mainly include cutaneous vasculitis, gouty arthritis, herpes zoster and diabetic foot ulcer (DFU). After many studies on its pharmacological effects, it was found that RHP can inhibit bacterial infection, increase lysosomal content, enhance immune defenses and inhibit inflammation. The traditional preparation is through the addition of honey to the powder, which is then directly applied to the affected area to treat diseases;

however, the formulation has been innovated using the original preparation through the continuous implementation of modern technology and made into creams, cataplasms, films, and sponges[2,3].

DFU is a diabetic complication. Its pathogenic causes are often vascular and nerve lesions, resulting in lower limb infection and the formation of foot ulcers[4]. The clinical manifestations are ischemic necrosis or damage to skin tissue, incomplete skin, wound exudation, abscess generation, *etc*. From the perspective of traditional Chinese medicine, DFUs are caused by deficiency of Qi and Yin, weakness of pulse, and blockage of dampness-heat and blood stasis toxin. Traditional chinese medicine also has shown promising effects with regard to safety and renoprotection in some prospective, multicenter, randomized, controlled clinical trial conducted[5]. And studies have found that RHP has a good effect in the treatment of DFUs in traditional Chinese medicine. Shao *et al*[6] found that its combination with antibiotics can quickly reduce the swelling of patients' ulcers to shorten the course of treatment. Liu *et al* [7] treated 40 patients with diabetic skin abscesses with the external application of RHP and found that the rate of effective treatment in the treatment group was higher than the control group. This result shows that RHP has a therapeutic effect on DFU. Zhang[8] treated patients with RHP and Simiao Tongluo decoction and found that this prescription can promote wound healing to promote improvement and play a therapeutic role.

Gas chromatography-mass spectrometry (GC–MS) and ultra-performance liquid chromatography-mass spectrometry (UPLC–MS) are widely used in the separation and identification of complex components, of which GC–MS is mainly used to separate the sample into volatile products in the instrument by pyrolysis of the sample at high temperature, with the advantages of high sensitivity, large information content, high efficiency, and small sample requirements. UPLC–MS technology allows the sample to be separated in the mobile phase after the ionization process through fragment ion mass number analysis and identification. This technology can compensate for the disadvantages of GC–MS, which cannot analyze components with features such as strong polarity, thermal instability, and difficult volatilization; UPLC-MS has the advantages of low detection limit, high automation, wide analysis range, and short analysis time. Network pharmacology research is mainly based on databases and software to obtain important target information for drug treatment of diseases and establish network connections to predict its mechanism of action. Because network pharmacology analysis shows synergy and compatibility with traditional Chinese medicine in the treatment of diseases and the therapeutic principle of syndrome differentiation and treatment, it is widely used in traditional Chinese medicine to explore the mechanism of action in the treatment of diseases from multiple targets. Wound healing is caused by a variety of molecular proteins that affect cells and remodel the tissue. Hematoxylin-eosin (HE) staining electron microscope observation is a more intuitive way to observe the healing of the wound surface. Fibroblasts can be clearly observed to evaluate drug treatment.

Although it has been proven that RHP has a therapeutic effect on DFU, the specific mechanism of action is not clear. In this study, the chemical components of RHP were extracted by supercritical extraction and alcohol extraction and separated and identified by GC–MS and UPLC–MS techniques, respectively, to obtain the active ingredients of the RHP formula, and the target pathway for the treatment of DFU was studied using network pharmacological analysis. In addition, a rat model of DFU was established to verify the therapeutic effect of RHP on wound healing, providing a direction for further clinical research.

MATERIALS AND METHODS

Materials

RHP (201202056, Jilin Shuangshi Pharmaceutical Co., Ltd) was purchased from Harbin Shiyitang Chinese Herbal Medicine Co., Ltd. (Harbin, China). Methanol (purity) and acetonitrile (purity) were all obtained from Merck Co., Inc. (Germany). Formic acid (purity) was obtained from West Asia Chemical Technology Co., Ltd. All other chemicals and solvents were of analytical grade.

Animals and drug administration

Thirty healthy male Sprague Dawley rats (weights, 200 ± 20 g) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan, China) and housed for adaptive feeding for 2 d. The animal facilities and protocols were approved by the Animal Ethics Committee of Heilongjiang University of Chinese Medicine (Heilongjiang, China).

Thirty rats were divided into 3 groups with 10 rats in each group, including the blank, model and RHP groups. The model and RHP groups were injected with streptozotocin/0.1 mol·L^{-1} citrate buffer solution (1/100, g/v). After 72 h, fasting blood glucose was measured with an electronic blood glucose meter (Sannuo Biosensor Co., Ltd, China). The modeling standard was that the random blood glucose level was greater than 12.0 mmol/L, accompanied by the typical symptoms of diabetes mellitus. In the establishment of the ulcer model, the rat hindfoot skin in each group was cut with scissors (the depth of the wound that would reach the fascia). The wound was cleaned before each treatment every day. The wound area was measured on the 5th, 7th, and 14th days, and the wound-healing rate was calculated. The formula for the healing rate was as follows: Healing rate (%) = (original wound area-unhealed

area)/original wound area × 100%.

After 14 d, the rats were sacrificed, and the skin around the wound was cut into 3 cm × 3 cm areas. The cut tissue was fixed with a 2.5% pentanediol solution and stored at low temperature (-80 °C).

The sample tissues were placed in liquid paraffin, freeze-fixed and sliced. Dewaxing, hydration and dyeing with hematoxylin staining solution and eosin dye solution were performed. The samples were air dried, sealed and observed with transmission electron microscopy (HT7700, Hitachi, Japan).

Composition analysis of RHP by GC–MS

Supercritical fluid extraction (SFE) equipment (HA220-40-11, Nantong Huaan Supercritical Extraction Co., Ltd, China) was used to extract volatile oil. First, an appropriate amount of RHP sample was crushed and sifted through a 40 μm mesh; the medicinal material was placed into supercritical extraction equipment, and the pressure was boosted to the set parameters for extraction. The pressure of the extraction kettle was 25 MPa, and its temperature was 45 °C. The pressure of the separating kettle was 8 MPa, and its temperature was 60 °C. The pressure of the other separating kettle was 4.5 MPa, and its temperature was 37 °C. The pump frequency of the SFE was 18 Hz, and the flow rate was 60 L/h. After extraction, the materials were removed to obtain the SFE.

GC–MS (5975B, Agilent Technologies, Inc., United States) was used to analyze the chemical composition of the SFE extraction. The prepared SFE extract was vortexed for 2 min, extracted with a solid-phase microextraction needle for 20 min, centrifuged for 20 min, and filtered with a membrane. Each of the samples was injected into GC–MS equipment equipped with an HP-INNOWAX (25 m × 0.20 mm, 0.40 μm) column (Agilent, United States) at 100 °C for 5 min. Then, the temperature was raised to 150 °C at 5 °C/min and then to 280 °C at 30 °C/min. The inlet temperature was 240 °C, and the flow rate of the carrier gas was 1.0 mL/min. The ion source temperature of MS with an EI source was 200 °C, and its transmission line temperature was 250 °C. The bombardment voltage was 70 eV.

Composition analysis of RHP by UPLC–MS

UPLC–MS (Ultimate 3000LC, Q Exactive HF, Thermo Fisher, United States) was used to analyze the chemical composition of RHP. First, the sample was pulverized, passed through a 40-μm mesh sieve, accurately weighed and placed in a stoppered conical flask. Hydrochloric acid/ethanol solution (1/100, v/v) was accurately added and ultrasonically treated for 40 min. The filtrate was shaken well, filtered and diluted into a 50-mL volumetric flask to obtain a sample solution. The reference substance was precisely weighed and placed in a 10-mL volumetric flask; methanol solution was added, and the solution was diluted to volume after ultrasonic treatment and shaken well to obtain the reference substance solution. Each of the samples was injected into UPLC–MS equipment equipped with a C18 chromatographic (2.1 mm × 100 mm, 1.8 μm) column (Zorbax Eclipse, Agilent, United States) at 30 °C. The flow rate was 0.3 mL/min. The mobile phase was water/formic acid (0.1/100, v/v) (A)/acetonitrile (B). The injection volume was 2 μL.

Mass spectrometry conditions utilized positive and negative modes for UPLC–MS. Electrospray ionization was used in ionization mode with a sheath gas flow rate of 45 arb. The auxiliary gas flow rate was 15 arb. The purge gas flow rate was 1 arb. The electrospray voltage was 3.5 KV. The capillary temperature was 330 °C. The S-Lens RF level was 55%. The scan mode was full scan/dd-MS2 (TopN = 10) with a scanning range of 100-1500 m/z and a resolution of 120000/60000. The collision mode was high energy collision dissociation.

Building a drug-component-target-disease network relationship graph

Cytoscape (v3.7.2.) is an analysis software that shows the complex corresponding relationship of "drug-component-target-disease" in the form of a network graph. It can conveniently visualize a network relationship, perform network topology analysis, analyze the degree of connection between each node according to the relevant parameters, and thus enable researchers to draw the corresponding conclusions.

Determining the "Chemical Composition-Target" correspondence

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (http://Lsp.nwu.edu.cn/) is a platform for the analysis and study of traditional Chinese medicines in many aspects, and it synthesizes the chemical composition and drug target data of traditional Chinese medicines[9], closely links diseases with targets and components, and explains the mechanism of action of traditional Chinese medicines[10]. The chemical components extracted from ten traditional Chinese medicines of the RHP formula were searched by entering the CAS number of chemical components in the TCMSP database, and the information related to the components could be obtained, of which the "Relatedtarget" column contained the targets corresponding to the component, and the obtained targets were integrated to determine the corresponding relationship between the components and the target.

Screening targets of RHP

Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/) is a database that brings together detailed information on the intersection between genes, proteins and diseases[11], and the combination

Table 1 Statistical analysis of wound healing rate at different times

Time	0 d (%)	3 d (%)	7 d (%)	14 d (%)
Control group	0	10.41 ± 0.24	17.43 ± 0.13	33.73 ± 0.28
Experimental group	0	32.78 ± 0.46	38.14 ± 0.28	53.11 ± 0.07
Homogeneity of variance	–	√	√	√
P value	–	< 0.05	< 0.05	< 0.05

of these data with that of their pathways and functions can further elucidate the mechanism of diseases [12]. A component usually corresponds to one or more targets, and information unrelated to the treatment of DFU in the above integrated "component-target" dataset needs to be screened out. In the TCMSP database, the information related to each target in the "Relatedtarget" column above was viewed, the relevant target with the keywords "diabetes-related diseases", "pain" and "bacterial infection" was selected according to the disease type in "Relateddiseases", and its "GenecardID" was recorded. To prevent the limitations of the TCMSP database on the target and disease correspondence and make the study more accurate, the targets without keywords were searched in the CTD database to further determine whether they were related to DFU in the "Diseases" category. Two databases, TCMSP and CTD, were used to screen for and obtain the chemical compositions of targets related to DFU.

Molecular docking of ingredients and targets

DiscoveryStudio (DS v19.1.0) software is a life science molecular simulation software that can be used to establish molecular docking models. Cytoscape was used to visualize the targets and components, analyze the key targets and important components, perform molecular docking, and observe the binding effect of chemical components to target proteins. First, the structural model of the component was downloaded from the TCMSP database; the human protein number corresponding to the target from the UniProt database was queried, and the number from the PDB database was the input to obtain the three-dimensional structural model of the target protein. The component structure and protein structure were opened in DiscoveryStudio software; H_2O and ligand were removed, the protein was hydrogenated, and the relationship between the molecule and the protein was established. In the end, the binding sites can be identified.

Pathway enrichment analysis

The STRING database (https://cn.string-db.org/) and the DAVID database (https://david.ncifcrf.gov) are mainly used to provide information on the individual target genes and proteins or the interaction between multiple histones and to perform GO or KEGG pathway enrichment analysis on them. The STRING database was used to observe the correlation between target proteins of RHP formula herbs, and the DAVID database was used to enrich KEGG pathways for target components and targets to obtain possible pathways for the treatment of DFU.

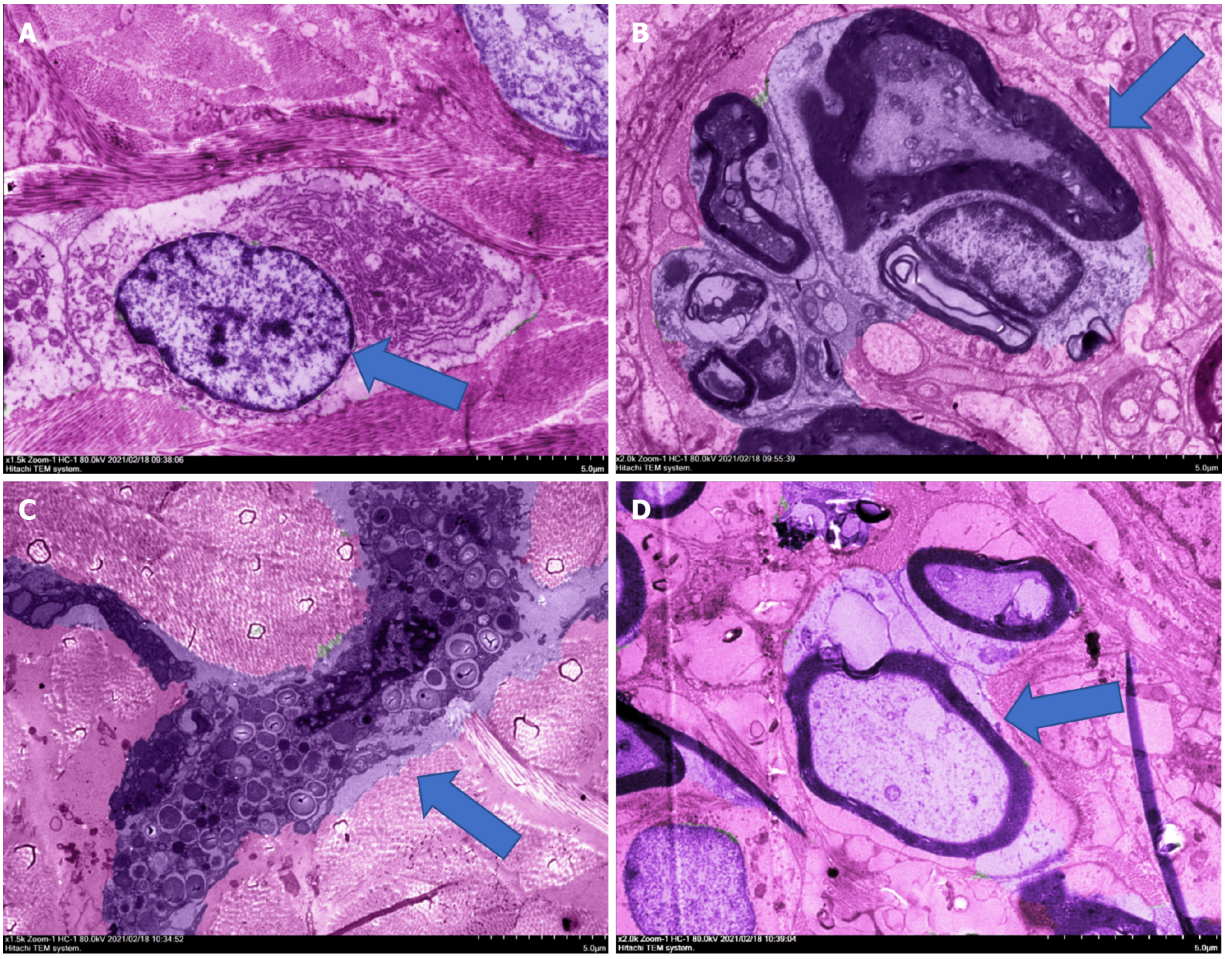
Statistical analyses

SPSS software (Version 24.0.0, Chicago, IL, United States) was used to analyze the wound-healing rate results of rats at different times, and all data are expressed as the mean ± SD. The differences were considered significant at $P < 0.05$.

RESULTS

In vivo wound healing effect

During continuous culture for 14 d, the wound areas of the rats on the first, third, seventh, and fourteenth days were recorded, and the cure rate was calculated. The results are shown in Table 1; the difference was significant. The results of the three groups of experiments showed that the cure rate of the RHP group was increased and reached 53.11%, indicating that RHP has a good therapeutic effect on the healing of DFU wounds in rats. The HE staining electron microscope results showed that after 14 d of uninterrupted administration, the ulcer tissues of 10 rats in the model group showed varying degrees of demyelination changes, unlike rats in the blank group, which showed symptoms of neuritis, as shown in Figure 1. In addition, neutrophil infiltration and granulation tissue loosening were observed. The mice treated with powder had good fibroblast function, mature granulation tissue, and restored nerve cells. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes were observed, but there were a few mast cells. RHP promoted the healing of DFUs in rats by affecting fibroblasts and nerve cells.



DOI: 10.4239/wjd.v13.i8.622

Figure 1 Hematoxylin-eosin staining electron micrograph of rat diabetic foot ulcer. A: The fibroblasts of blank group; B: The neuritis of model group; C: Mast cells of powder group; D: Nerve cells of powder group. The mice treated with powder had good fibroblasts function, mature granulation tissue, and restored nerve cells. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes would be observed, but there were a few mast cells. Ruyi Jinhuang powder promoted the healing of diabetic foot ulcers in rats by affecting fibroblasts and nerve cells.

Composition analysis of RHP by GC–MS

The GC–MS full spectrum identification results of RHP are shown in Table 2, and a total of 43 components were detected to obtain the total ion flow diagram (Figure 2), which mainly included alcohols, enes, esters, phenols, and other components, accounting for 27.91%, 20.93%, 20.93%, 16.28%, 4.65%, and 9.30% of the total, respectively. Among them, the higher components were ar-turmerone 24.33% (No. 23), tigerone 20.74% (No. 17), agarospirol 13.80% (No. 18), β-eudesmol 9.06% (No. 22), palmitic acid 2.31% (No. 43), and (E)-allylantone 2.23% (No. 29).

Composition analysis of RHP by UPLC–MS

The UPLC–MS full spectrum identification results of RHP are shown in Table 3. A total of 46 components were detected. The main components include flavonoids, organic nitrogens, isopentenol esters, carboxylic acids and their derivatives, benzene and its substituted derivatives, diarylheptans, fatty acyls, anthracyclines and other components, accounting for 23.92%, 15.23%, 10.88%, 8.71%, 6.52%, 6.52%, 6.52%, 4.26% and 17.44% of the total, respectively. The positive and negative ion flow diagrams are shown in Figure 3. Among them, the components with the highest levels were α,α-trehalose (409.04%; No. 6), curcumin (307.20%; No. 37), berberine (232.25%; No. 24), (3R,5R)-1,3,5-trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid (201.34%; No. 16), bisdemethoxycurcumin (153.92%; No. 33), genistein (153.83%; No. 39), demethoxycurcumin (151.99%; No. 35), and citricol acid (145.29%; No. 9).

Establishment and analysis of the network relationship of "Traditional Chinese Medicine-Ingredients-Target"

Eighty-nine components were obtained by GC–MS and UPLC–MS. According to the "target-disease" relationship in the database, 43 relevant components for the treatment of DFU were finally screened

Table 2 Gas chromatography–mass spectrometry full spectrum identification results

No.	RT (min)	Relative content (%)	Qualitative	CAS	Name
1	26.36	0.59	51.56	512-61-8	(-)-α-Santalene
2	27.07	0.26	25.81	87-44-5	Caryophyllene
3	29.71	1.86	65.77	495-60-3	α-Zingiberene
4	29.83	0.64	14.58	495-61-4	6-methyl-2-(4-methylcyclohex-3-enyl)hept-1,5-diene
5	30.64	1.95	60.85	20307-83-9	β-Sesquiphellandrene
6	30.7	1.33	80.1	644-30-4	α-Curcumene
7	34.21	0.38	26.47	56192-70-2	(Z)-a-Atlantone
8	34.27	0.23	61.92	1139-30-6	Caryophyllene oxide
9	34.36	0.30	19.52	83173-76-6	2-Methyl-1-(4-methylphenyl)-3-buten-1-ol
10	34.52	0.25	58.19	108549-47-9	(6Z)-2-Methyl-6-(4-methyl-3-cyclohexen-1-ylidene)-2-hepten-4-one
11	35.47	0.58	29.36	639-99-6	Elemol
12	35.78	0.28	58.71	145512-84-1	Trans-Sesquisabinene hydrate
13	36	0.37	62.02	58334-55-7	Zingiberenol
14	36.57	0.74	86.3	6989-21-5	Atractylon
15	36.83	0.25	12.5	82508-14-3	2-methyl-6-(4-methylidenecyclohex-2-en-1-yl)hept-2-en-4-one
16	37.23	0.77	22.65	1209-71-8	γ-Eudesmol
17	37.41	20.74	91.81	180315-67-7	Tumerone
18	37.67	13.80	22.04	1460-73-7	Agarospirol
19	37.81	1.75	38	112-39-0	Methyl palmitate
20	38.41	1.26	41.2	473-16-5	a-Eudesmol
21	38.48	0.72	9.97	515-20-8	Costol
22	38.65	9.06	76.48	473-15-4	β-Eudesmol
23	39.1	24.33	97.75	532-65-0	ar-Turmerone
24	39.28	0.24	11.67	19888-00-7	4,8-Cycloundecadien-1-ol, 6,6,9-trimethyl-2-methylene-, (1R,4E,8E)-
25	39.47	0.23	4.42	65646-68-6	N-(4-hydroxyphenyl)retinamide
26	39.64	0.39	8.22	19912-67-5	(+)-Epicubenol
27	39.9	0.85	49.47	30666-87-6	2-Methyl-6-(4-methylphenyl)-4-heptanone
28	40.16	1.05	49.07	72441-71-5	(6R,7R)-Bisabolone
29	40.53	2.23	89.2	108645-54-1	(E)-Atlantone
30	40.89	0.65	82.79	120681-80-3	2-Cyclohexene-1-propanol,g-methyl-4-methylene-a-(2-methyl-1-propen-1-yl)
31	41.58	0.45	75.15	112-61-8	Octadecanoic acid, methyl ester
32	41.88	1.87	16.03	112-62-9	Methyl oleate
33	41.98	0.65	7.88	1937-62-8	trans-octadec-9-enoicacidmethylester
34	42.55	1.03	26.03	112-63-0	methyl linoleate
35	43.54	0.42	42.85	301-00-8	9,12,15-Octadecatrienoic acid, methyl ester
36	43.61	0.22	4.63	29550-55-8	Teresantalol(7CI)
37	44.58	0.24	88.5	69301-27-5	2-(1,5-Dimethyl-4-Hexenyl)-5-Methyl-Phenol
38	43.85	0.84	6.23	38142-57-3	2-Methyl-6-(p-tolyl)hept-2-en-4-ol
39	45.01	1.11	28.14	3218-36-8	4-Biphenylcarboxaldehyde
40	46.86	0.84	75.64	4666-84-6	Proximadiol
41	47.75	0.46	98.07	949081-10-1	(S)-3-Methyl-6-((S)-6-methyl-4-oxohept-5-en-2-yl)cyclohex-2-enone

42	48.71	0.24	9.54	2566-90-7	Methyl 4,7,10,13,16,19-cis-docosahexanenoate
43	51.08	2.31%	83.12	1957-10-3	Palmitic acid

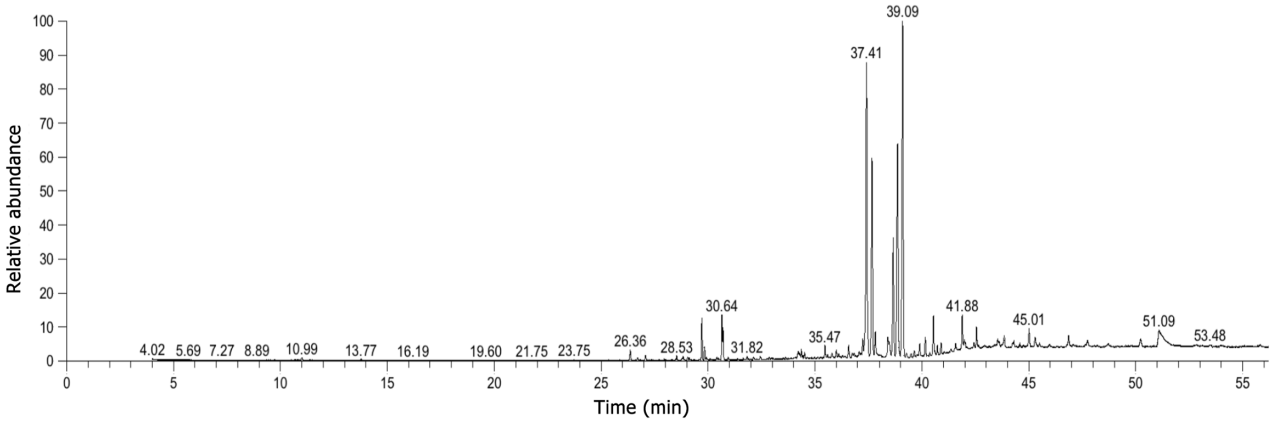


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Figure 2 The total ion flow diagram of gas chromatography–mass spectrometry. Among them, the higher components were ar-turmerone 24.33%, tumerone 20.74%, agarospirol 13.80%, β-eudesmol 9.06%, palmitic acid 2.31%, (E) atlantone 2.23%, β-quadruphellandrene 1.95%, methyloleate 1.87%, α-zingiberene 1.86%, and methylmitate 1.75%.

from the ten medicines. There were 36 related targets. The results are shown in Table 4. Cytoscape software was used to visualize the network relationship of "TCM-Ingredient-Target", which was constructed by the parameters of "Degree", "Betweenness" and "Closeness" of each node, as shown in Figure 4. The important medicinal materials of RHP for the treatment of DFU are *turmeric, Magnolia officinalis*, and *rhubarb*, and the important components are luteolin, naringin, demethoxycurcumin, gallic acid, methyl linoleate, caryophyllene, arylcurcumin, methyl palmitate, berberine and rheic acid. The important targets are posttranscriptional silencing (PTGS2), muscarinic acetylcholine receptor M2 (CHRM2), Dipeptidyl peptidase 4, topoisomerase II alpha (TOP2A), retinoic X receptor alpha (RXRA), Protein tyrosine phosphatase nonreceptor type 1, tumor necrosis factor (TNF), interleukin 6 (IL-6), *etc.*

Analysis of the docking results of important components and key target molecules

Molecular docking was performed between important components of luteolin, methyl palmitate, ar-turmeric, methyl linoleate, palmitic acid, demethoxycurcumin, and naringin and the key targets PTGS2, TOP2A, CHRM2, and RXRA. The results are shown in Table 5, in which LibDockScore indicates the docking effect, and the value indicates the docking effect. Seven components (luteolin, methyl palmitate, ar-turmeric, methyl linoleate, palmitic acid, demethoxycurcumin and naringin) had a large LibDockScore, indicating that the binding effect between these components and key targets was good. The component docking conformations shown in Figure 5 indicate the following: The complex naringin-PTGS2 was stabilized by five conventional hydrogen bonds, eight carbon-hydrogen bonds, and two bonds to the alkyl with residues including GLY, ANS, ARG, PHE, ASN, HIS, GLY, GLN, LEU; the complex methyl linoleate-RXRA was stabilized by three carbon-hydrogen bonds, two Pi bonds to the alkyl with residues including DA and DG; the complex luteolin-TOP2A was stabilized by four conventional hydrogen bonds, two carbon-hydrogen bonds, two Pi bonds to the alkyl, six Pi bonds to the benzene ring with residues including LYS, GLU, SER, and ASP; the complex naringin-TOP2A was stabilized by three conventional hydrogen bonds, four carbon-hydrogen bonds, one Pi bond to the alkyl, four Pi bonds to the benzene ring with residues including ASP, ARG, ASN, and LYS; the complex methyl palmitate-PTGS2 was stabilized by two conventional hydrogen bonds, one Pi bond to the alkyl with residues including VAL, ASN, and TRP; the complex ar-turmeric-CHRM2 was stabilized by nine Pi bonds to the alkyl, two Pi bonds to the benzene ring and Van der Waals interactions with residues including PHE, LYS, ILE, VAL, LEU, ASP, ARG, ASN, and THR; the complex methyl linoleate-PTGS2 two carbon-hydrogen bonds, one Pi bond and one bond to the alkyl with residues including VAL, ASN, ILE, and TRP; the complex ar-turmeric-PTGS2 was stabilized by one conventional hydrogen bond, one Pi bond and three bonds to the alkyl, two Pi bonds to the benzene ring with residues including GLN, PHE, VAL, PRO, and ASN; the complex palmitic acid-PTGS2 was stabilized by two conventional hydrogen bonds with residues including VAL and GLY; the complex demethoxycurcumin-PTGS2 was stabilized by four conventional hydrogen bonds, one carbon-hydrogen bond, three Pi bonds to the benzene ring with residues including GLY, VAL, ASN, and PHE.

Table 3 Ultra-performance liquid chromatography full spectrum identification results

No.	Name	CAS	Molecular weight	RT (min)	Relative concentration (μg/mL)
1	DL-Arginine	7200-25-1	174.11175	0.783	24.44253696
2	Nitrosobis(2-oxopropyl)amine	60599-38-4	158.06914	0.817	15.74503993
3	Gluconic acid	526-95-4	196.05765	0.821	82.48616363
4	D-(+)-Proline	344-25-2	115.06357	0.846	15.35704499
5	Cabotegravir	1051375-10-0	405.11182	0.847	20.08010135
6	α,α-Trehalose	99-20-7	342.11623	0.847	409.0449144
7	D-(-)-Quinic acid	77-95-2	192.06275	0.85	56.2670819
8	Isocitric acid	320-77-4	192.02637	0.939	63.29259087
9	Citric acid	77-92-9	192.02637	1.169	145.2856943
10	Gallic acid	149-91-7	170.02078	1.899	23.57871448
11	Chlorogenic acid	327-97-9	354.09527	5.332	44.58986172
12	Catechin	88191-48-4	290.07917	5.431	98.07463825
13	methyl chlorogenate	123483-19-2	368.11081	5.501	18.15074983
14	6-Acetylcodeine	6703-27-1	341.16237	5.886	12.3548527
15	2-(3,4-Dihydroxyphenyl)ethyl 3-O-(6-deoxy-β-L-mannopyranosyl)-6-O-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]-β-D-glucopyranoside	61303-13-7	624.20617	6.092	20.78761602
16	(3R,5R)-1,3,5-Trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid	2613-86-7	368.11084	6.196	201.3429867
17	Emodin-8-Beta-D-Glucoside	23313-21-5	432.10571	6.579	7.280885459
18	2-[4-[3-[3,4-dihydroxy-4-(hydroxymethyl)oxolan-2-yl]oxy-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-7-hydroxy-2,3-dihydrochromen-4-one	74639-14-8	550.16902	6.625	13.98565399
19	Baicalin	21967-41-9	446.0852	6.663	16.85569046
20	Liquiritin	551-15-5	418.12676	6.737	22.91985259
21	Naringin	10236-47-2	580.17964	6.959	26.36327029
22	Hesperidin	520-26-3	610.19038	7.191	85.82076776
23	Azelaic acid	123-99-9	188.1042	7.548	4.773123466
24	Berberine	2086-83-1	335.11525	8.242	232.2489146
25	isosakuranetin-7-O-rutinoside	14259-47-3	594.19556	8.493	4.34803523
26	Tinnevellin glucoside	80358-06-1	408.14228	8.786	13.59498227
27	Daidzein	486-66-8	254.05778	9.009	13.05981793
28	Chrysophanol 8-O-β-D-glucoside	13241-28-6	416.11099	9.01	17.88080565
29	Licoricesaponin G2	118441-84-2	838.39998	9.824	7.195669531
30	Luteolin	491-70-3	286.04776	9.909	1.034661042
31	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	95341-44-9	330.24071	9.938	19.41925634

32	Rheic acid	478-43-3	284.03211	11.029	81.8044913
33	Bisdemethoxycurcumin	24939-16-0	308.10496	11.328	153.9247824
34	6-Hydroxy-2-naphthoic acid	16712-64-4	188.04652	11.328	7.047135466
35	Demethoxycurcumin	22608-11-3	338.11567	11.473	151.9906609
36	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-methylbut-2-enyl)-2,3-dihydro-chromen-4-one	76735-58-5	370.14168	11.541	55.08087646
37	Curcumin	458-37-7	368.12606	11.619	307.1972754
38	Isoimperatorin	482-45-1	270.08918	11.847	1.025466018
39	Genistein	446-72-0	270.05279	12.256	153.8276373
40	(+/-)9-HODE	98524-19-7	296.23511	13.146	23.43829567
41	(+)-ar-Turmerone	532-65-0	216.15135	13.605	23.84749189
42	Indane	496-11-7	118.07843	13.605	27.63249372
43	Prespatane	100387-71-8	204.18777	13.956	2.010509748
44	(+)-Nootkatone	4674-50-4	218.16696	14.497	16.6606139
45	Aristolone	6831-17-0	218.16696	14.588	45.88543598
46	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	119-47-1	340.24015	16.788	25.70628216

RT: Radiotherapy.

Analysis of KEGG pathway enrichment results

The interaction between each target protein was preliminarily obtained by entering the target protein into the STRING database, as shown in Figure 6. Among the 36 targets, except for CHRM2, CHRM4, PCYT1A and Sodium voltage-gated channel alpha subunit 5, the remaining 32 target proteins are closely related to each other and may participate in multiple pathways. The target list was uploaded to the DAVID database to establish the "pathway-target" link. A total of 27 pathways related to DFU treatment were retrieved. The number of targets contained in each pathway is shown in Figure 7A. Cytoscape was used to construct the "pathway-target" network relationship diagram, which is shown in Figure 7B; the "pathway-target" relationship is shown in Figure 7C. Correspondence between channel categories is shown in Figure 7D. Most of the 27 pathway diagrams belong to the immune system and participate in the processes of inflammatory and infectious diseases, which demonstrate signal transduction effects. This finding shows that RHP can affect the body's immune system, regulate inflammation through signal transduction, and exert curative effects in DFU. Further analysis of the literature shows that RHP is mainly related to the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) signaling pathway, neuroactive ligand–receptor interaction and the forkhead box O (FoxO) signaling pathway in the treatment of DFU.

We established the network relationship between the three pathways of the PI3K-Akt signaling pathway, neuroactive ligand–receptor interaction and FoxO signaling pathway and the target and chemical components and analyzed the topological parameters of each node. The network diagram (Figure 8) showed that CHRM2 and RXRA are the common targets of the three pathways.

The prediction of anti-DFU mechanism

According to the literature, wound healing mainly involves the three processes of hemostasis and inflammation, proliferation, and remodeling. The target pathways involved are diverse and complex, as shown in Figure 9. RHP can act on leukocytes by regulating IL-6 and TNF, regulating the inflammatory response, and then participating in hemostasis to participate in inflammation and remodeling stages. RHP can also affect platelets, macrophages and fibroblasts by acting on the PI3K-Akt pathway to promote angiogenesis participation in the proliferation stage and facilitate wound healing in DFUs.

Further analysis of the effects of the three pathways in the human body found that if the PI3K-Akt signaling pathway is inhibited in diabetic neurons, neuronal apoptosis is increased, and diabetic neuropathic pain is induced; thus, activating this pathway can alleviate painful diabetic peripheral neuropathy. The abnormal expression or loss of genes related to the neuroactive ligand–receptor interaction pathway can cause neuropathy, such as with the downregulation of glial gene expression, which impairs nerve impulse conduction and increases the potential nerve involvement of systemic

Table 4 Ruyi Jinhuang powder "Chinese medicine-ingredients-target" correspondence

CAS	Name	GenecardID	Attribution
512-61-8	(-)-α-Santalene	PTGS2, CHRM2	JH
87-44-5	Caryophyllene	PTGS2, CHRM2	BZ, JH, CZ, ZHP, TNX
495-60-3	α-Zingiberene	PTGS2, DPP4	JH
20307-83-9	β-Sesquiphellandrene	PTGS2	JH
644-30-4	α-Curcumene	PTGS2, TOP2A, DPP4	JH
1139-30-6	Caryophyllene oxide	CHRM2, DPP4	ZHP, TNX
639-99-6	Elemol	CHRM2	ZHP, JH
58334-55-7	Zingiberenol	CHRM2	JH
6989-21-5	Atractylon	SCN5A, CHRM4, HTR2A, CHRM2, OPRM1	CZ
82508-14-3	2-methyl-6-(4-methylidenecyclohex-2-en-1-yl)hept-2-en-4-one	PTGS2, CHRM2	JH, ZHP, CZ
112-39-0	Methyl palmitate	PTGS2, IL-10, TNF, IL-6	JH, ZHP, BZ, HB, THF
473-15-4	beta-Eudesmol	CHRM2	ZHP, JH, CZ
532-65-0	(6S)-2-methyl-6-(4-methylphenyl)hept-2-en-4-one	DPP4, PTGS2, CHRM2x, ampC	JH
112-62-9	Methyl oleate	RXRA	ZHP, THF
1937-62-8	trans-octadec-9-enoicacidmethylester	RXRA	ZHP, THF
112-63-0	methyl linoleate	PTGS2, RXRA	BZ, THF, ZHP, TNX
301-00-8	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	PTGS2, RXRA	THF
38142-57-3	2-Methyl-6-(p-tolyl)hept-2-en-4-ol	PTGS2, CHRM2, DPP4, ampC, RXRA	THF, ZHP, TNX
1957—10—3	Palmitic acid	PTGS2, IL-10, TNF, PCYT1A	DH, ZHP, THF
149-91-7	Gallic acid	PTGS2, PTPN1, TOP2A	DH, TNX, CP, GC, JH
23313-21-5	Emodin-8-Beta-D-Glucoside	TOP2A	DH
458-37-7	Curcumin	PTGS2, PTPN1	JH
22608-11-3	Demethoxycurcumin	PTGS2, PTPN1, AKR1B1, ABCB1, SCN5A	JH
520-26-3	Hesperidin	PTGS2	CP
14259-47-3	isosakuranetin-7-O-rutinoside	TOP2A	CP
10236-47-2	Naringin	TOP2A, CDKN1A, TNF, RASGRF2, RAF, PTGS2, ampC, MTTP, APOB, mvaA, PPARA	CP, GC
21967-41-9	Baicalin	PTPN1, TOP2A	DH
88191-48-4	Catechin	CHRM2	DH, CP
491-70-3	Luteolin	PTGS2, DPP4, EGFR, IL10, CDK4, TNF, IL6, NFKBIA, psdA1, IL2, TOP2A, SLC2A4, INSR, MET	CP, ZHP
551-15-5	Liquiritin	PTGS2	GC
478-43-3	Rheic acid	PTGS2, AKR1B1	DH
2086-83-1	Berberine	PTGS2, RXRA	HB
77-92-9	Citric acid	PTGS2, AKR1B1, GRIN2A, GRIA2, PTPN1	DH
320-77-4	Isocitric acid	PTGS2	DH
7200-25-1	DL-Arginine	PTGS2	THF

446-72-0	Genistein	PTGS2, EGFR, TNF, SELE, IL8	GC
482-45-1	Isoimperatorin	PTGS2	BZ
486-66-8	Daidzein	PTGS2, RXRA	GC
6831-17-0	Aristolone	CHRM2, PTGS2	BZ
532-65-0	(+) -ar-Turmerone	PTGS2, RXRA, CHRM2, ampC, DPP4	JH
327-97-9	Chlorogenic acid	PTGS2	HB
77-95-2	D-(-)-Quinic acid	PTGS2	HB
1460-73-7	Agarospirol	CHRM2	ZHP

TNF: Tumor necrosis factor; IL: Interleukin; PTGS2: Posttranscriptional silencing; TOP2A: Topoisomerase II alpha; CHRM2: Muscarinic acetylcholine receptor M2; RXRA: Retinoic X receptor alpha; DPP4: Dipeptidyl peptidase 4; SCN5A: Sodium voltage-gated channel alpha subunit 5; HTR2A: 5-hydroxytryptamine (serotonin) receptor 2A; OPRM1: Opioid Receptor Mu 1; EGFR: Epidermal Growth Factor Recepto; PTPN1: Protein tyrosine phosphatase nonreceptor type 1; THF: Trichosanthin (Tian Hua); DH: Rhubarb (Da Huang); HB: Phellodendron (Huang Bai); JH: Turmeric (Jiang Huang); BZ: Angelica dahurica (Bai Zhi); TNX: Arisaema (Tian Nan Xing); CZ: Atractylodes lancea (Cang Zhu); HP: Magnolia officinalis (Hou Po); CP: Pericarpium Citri Reticulatae (Chen Pi); GC: Glycyrrhiza uralensis (Gan Cao).

Table 5 Docking parameters of chemical components and target protein molecules

Name	Target	LibDockScore
Luteolin	TOP2A	104.333
Methyl palmitate	PTGS2	114.221
ar-Turmeric	PTGS2	91.0625
	CHRM2	88.3765
Methyl Linoleate	RXRA	138.583
	PTGS2	130.838
Palmitic acid	PTGS2	114.349
Demethoxycurcumin	PTGS2	135.479
Naringin	TOP2A	141.197
	PTGS2	164.305

PTGS2: Posttranscriptional silencing; TOP2A: Topoisomerase II alpha; CHRM2: Muscarinic acetylcholine receptor M2; RXRA: Retinoic X receptor alpha.

diseases. The link between the FoxO signaling pathway and diabetes is that type II diabetes mellitus causes abnormal tissue signaling due to hyperglycemia or insulin resistance. The identified important targets, such as IL-6 and TNF, participate in the abovementioned pathways and play a role in hemostasis and tissue remodeling so that the DFU wound can heal. The mechanism of action is shown in Figure 10.

DISCUSSION

The results of the three groups of experiments showed indicated that RHP has a good therapeutic effect on the healing of DFU wounds in rats. The HE staining electron microscopy results showed neutrophil infiltration and granulation tissue loosening in the model group and blank group. The fibroblasts of the RHP group had good function, the granulation tissue was mature, and nerve cells were restored. The thickness of the stratum corneum and the integrity of the epidermis were good, showing a good state of recovery, and intact lymphocytes could be observed, but there were a few mast cells. Thus, RHP promoted the healing of DFUs in rats by affecting fibroblasts and nerve cells.

In this study, GC–MS and UPLC–MS were used to collect and identify the chemical components of RHP. Eighty-nine compounds were detected and analyzed, including flavonoids, terpenes, and coumarins. The components obtained by GC–MS are mainly volatile substances, and the components obtained by UPLC–MS are mostly high boiling point, nonvolatile, high molecular weight substances. The network pharmacology analysis of these compounds shows that RHP plays a role in the treatment

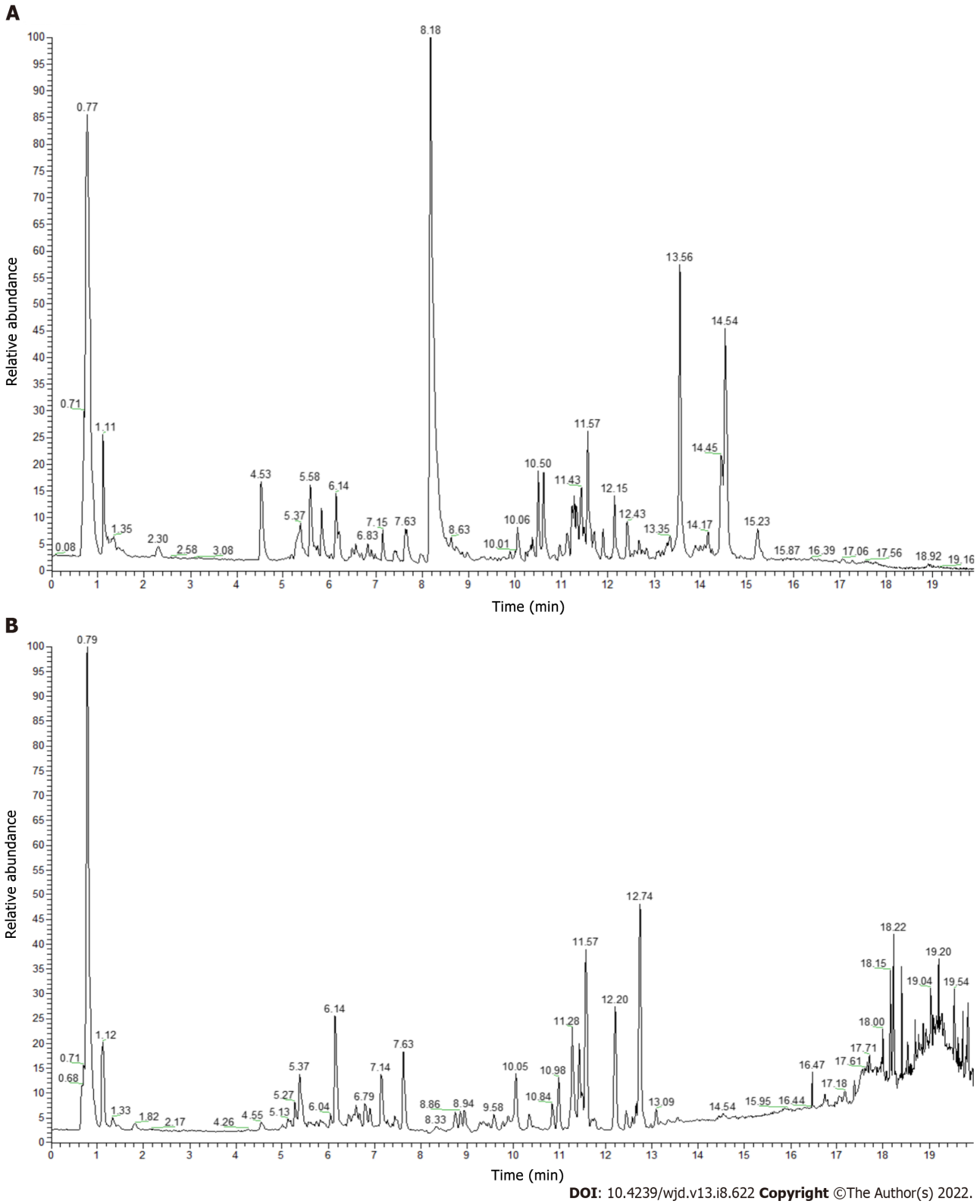

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Figure 3 Ultra-performance liquid chromatography ion flow diagram of Ruyi Jinhuang powder. A: Positive ion flow diagram; B: Negative ion flow diagram. Among them, the components with high levels were α,α-trehalose 409.04% (No. 6), curcumin 307.20% (No. 37), berberine 232.25% (No. 24), (3R,5R)-1,3,5-trihydroxy-4-{[(2E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoyl]oxy}cyclohexanecarboxylic acid 201.34% (No. 16), bisdemethoxycurcumin 153.92% (No. 33), genistein 153.83% (No. 39), demethoxycurcumin 151.99% (No. 35), and citricol acid 145.29% (No. 9).

of DFUs through multiple targets and channels. However, the manner in which specific components are combined with target proteins needs to be further explored.

DFU is one of the main complications of diabetes. Current studies have found that its main causes are neuropathy, vascular disease and impaired immune function[13,14]. The specific pathogenesis is that when persistent hyperglycemia occurs, nerve cells accumulate a large number of toxic components,

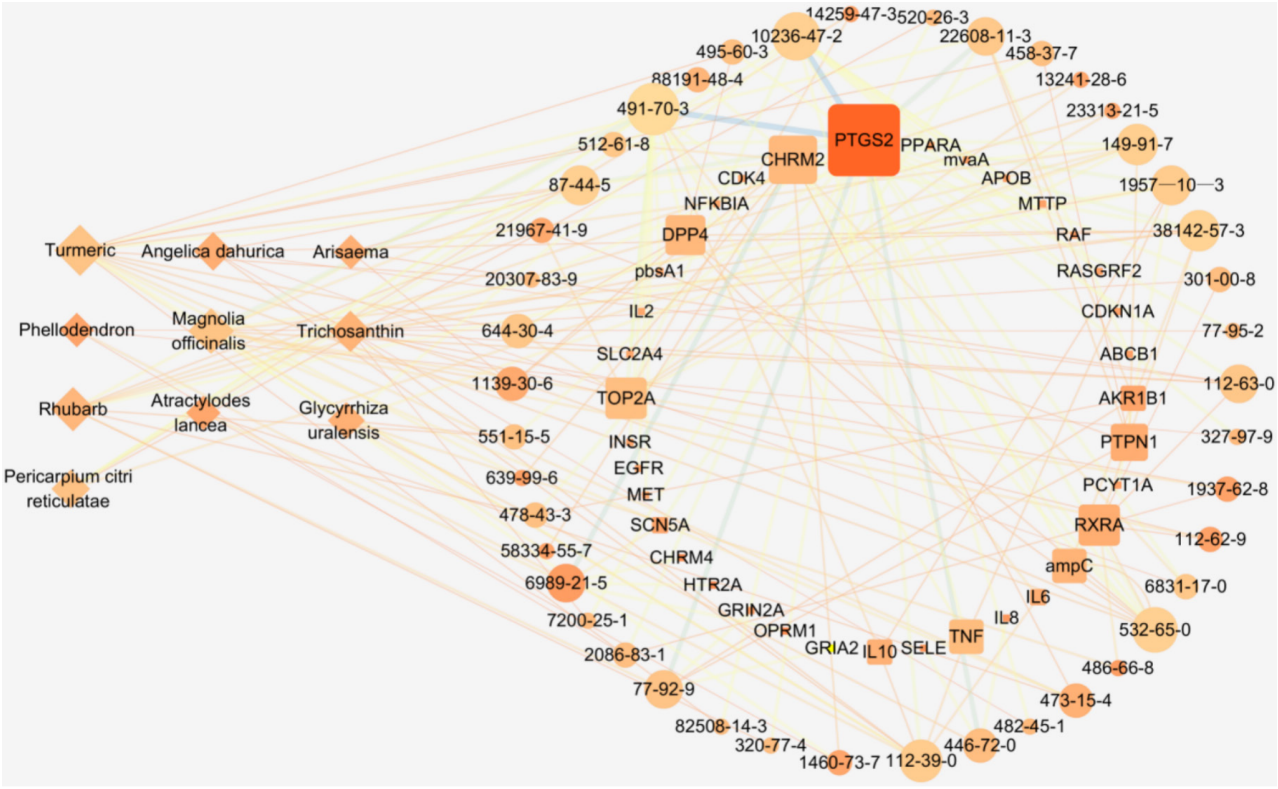

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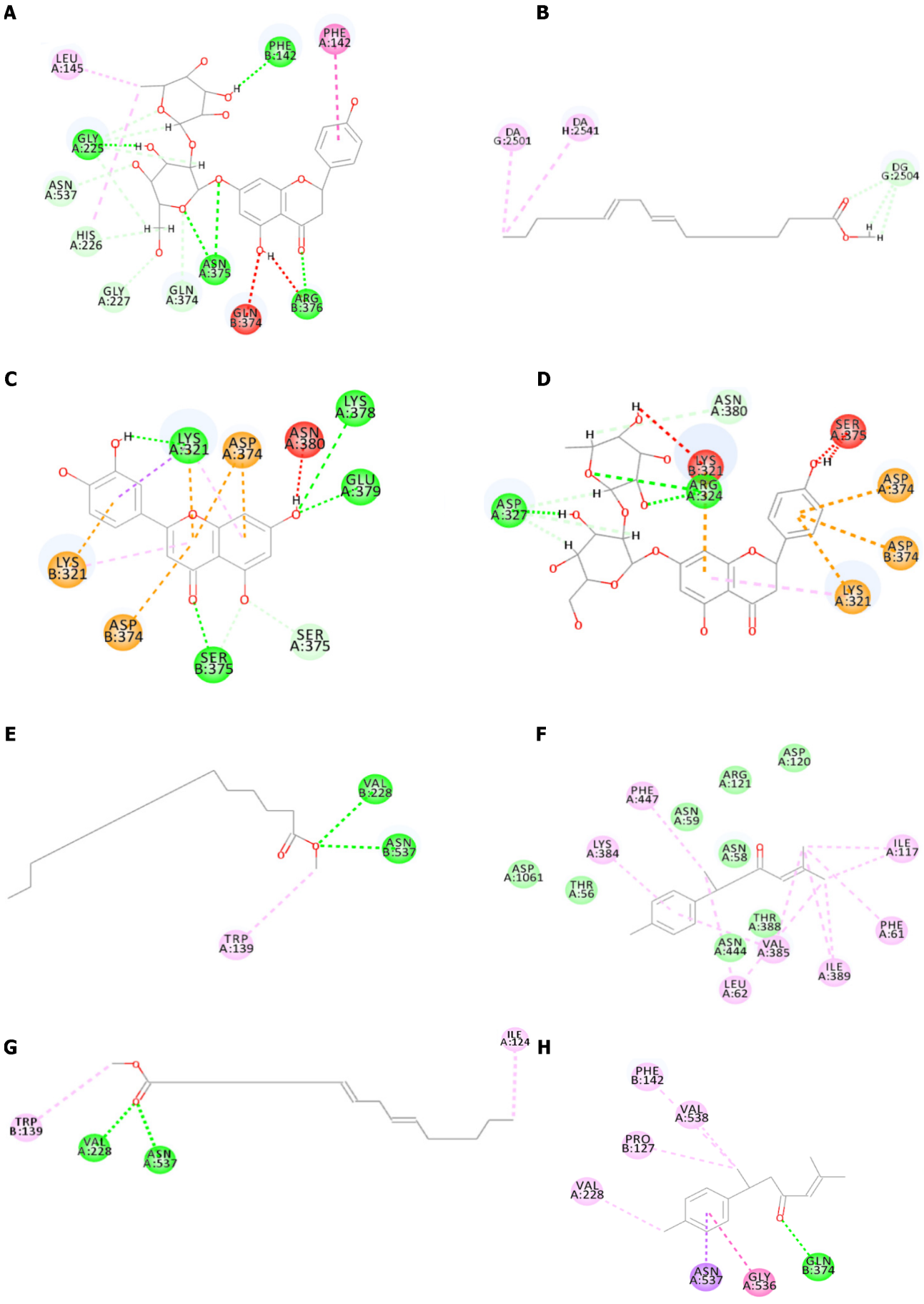
Figure 4 The "traditional Chinese medicine-ingredient-target" network of Ruyi Jinhuang powder topology diagram. There are a total of 89 nodes and 186 edges; diamonds represent ten herbs of Ruyi Jinhuang powder, circles represent 43 related chemical components (represented in the form of CAS number), squares represent 36 target targets (represented in the form of GenecardID), nodes indicate that the greater Degree value, the darker color indicates the greater Closness value, and the thicker lines indicate the greater Betweenness value.

damage to endothelial cell function occurs, vascular tension is decreased, and nerve ischemia occurs; thus, nerve and vascular lesions develop. In addition, persistent hyperglycemia affects the inflammatory response of the wound by acting on cells and the immune system, and the wound is persistently infected and difficult to heal[15,16].

The active ingredients were extracted from the ten herbs of RHP, and network pharmacological analysis of "herb-component-target pathway-disease" was performed to identify three key pathways: The PI3K-Akt signaling pathway; neuroactivated-receptor interactions; and the FoxO signaling pathway. These pathways are important targets for the treatment of DFU. Studies have found that RXRA has a negative regulatory effect on glucose-stimulated insulin secretion[17], while CHRM2 mainly plays a role in the central nervous system and peripheral nervous system and can activate endothelial cell NO lyase to relax vascular smooth muscle[18,19], which is a major cause of foot ulcers caused by diabetic vascular disease and is a key target for the treatment of vascular disease[20]. The mechanism of the PI3K-Akt signaling pathway in the treatment of DFU is reflected in the following two aspects. On the one hand, insulin initiates this signaling pathway by upregulating PI3K expression with Akt molecules and inhibits FoxO1 expression, thereby allowing normal glucose uptake by cells and improving abnormal lipid metabolism[21-23]. On the other hand, if the PI3K-Akt signaling pathway is inhibited in diabetic neurons, neuronal apoptosis is increased, and diabetic neuropathic pain is induced; thus, activation of this pathway can relieve painful diabetic peripheral neuropathy[24]. In addition, diabetic patients have difficulty healing repeated wound infections due to a prolonged wound inflammatory response due to metabolic disorders, and the PI3K-Akt signaling pathway can promote the expression of hypoxia-inducible Factor 1, an important factor in wound healing, to accelerate wound cell proliferation to promote healing[25]. The neuroligand-interreceptor interaction pathway is a collection of intracellular and extracellular related receptor ligands, and abnormal expression or deletion of related genes in this pathway can cause neuropathic lesions; for example, downregulation of Glral gene expression impairs nerve impulse conduction and increases the potential incidence of neurological diseases[26,27]. The link between the FoxO signaling pathway and diabetes is that type 2 diabetes induces skeletal muscle atrophy due to abnormal tissue signaling and protein disorders caused by hyperglycemia or insulin resistance, and insulin phosphorylates mediators of the FoxO signaling pathway and inhibits autophagy-related gene expression. Luteolin can reduce neuropathic pain by decreasing FoxO1 acetylation, regulating this pathway, and inhibiting the expression of inflammation- and pain-related genes[28]. In addition to these three pathways, the PI3K-Akt signaling pathway,


A
LEU A:145
PHE B:142
PHE A:142
GLY A:225
ASN A:537
HIS A:226
ASN A:375
GLY A:227
GLN A:374
GLN B:374
ARG B:376
B
DA G:2501
DA H:2541
DG G:2504
C
LYS A:378
LYS A:321
ASP A:374
ASN A:380
GLU A:379
LYS B:321
ASP B:374
SER B:375
SER A:375
D
ASN A:380
SER A:375
LYS B:321
ARG A:324
ASP A:374
ASP A:327
ASP B:374
LYS A:321
E
VAL B:228
ASN B:537
TRP A:139
F
ASP A:120
ARG A:121
PHE A:447
ASN A:59
LYS A:384
ASN A:58
ILE A:117
ASP A:1061
THR A:56
THR A:388
PHE A:61
ASN A:444
VAL A:385
ILE A:389
LEU A:62
G
ILE A:124
TRP B:139
VAL A:228
ASN A:537
H
PHE B:142
VAL A:538
PRO B:127
VAL A:228
GLN B:374
ASN A:537
GLY A:536

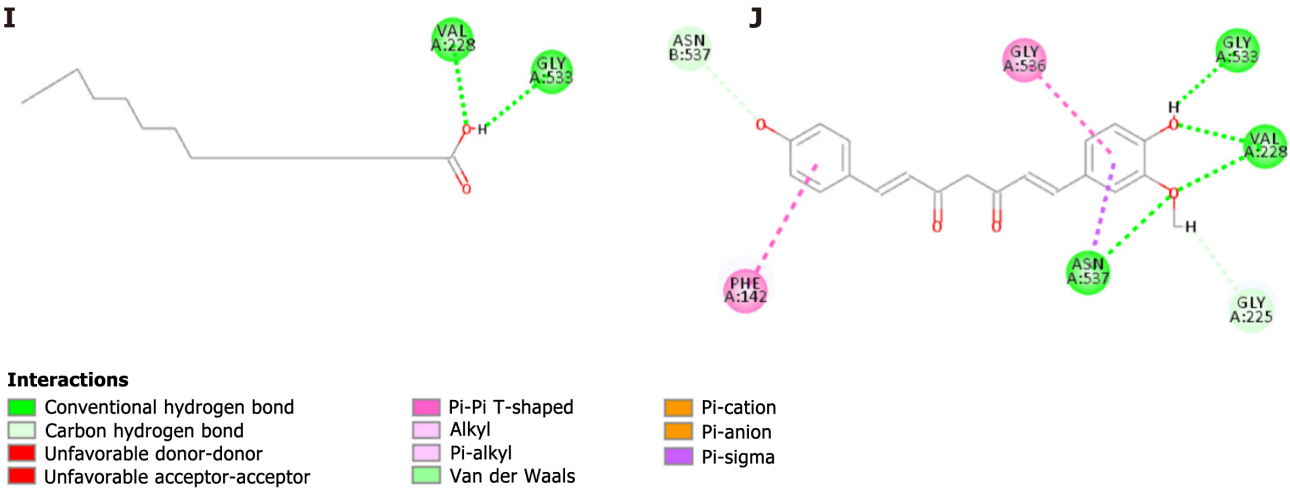


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Figure 5 The virtual docking of bioactive ingredients from Ruyi Jinhuang powder for diabetic foot ulcer targets. A and D: The virtual docking of naringin with post-transcriptionalgenesilencing (PTGS2) and Topoisomerase II alpha (TOP2A); B and G: The virtual docking of methyl linoleate with Retinoid X Receptor alpha and PTGS2; C: The docking of Luteolin and TOP2A; E: The docking of Methyl palmitate and PTGS2; F and H: The virtual docking of ar-Turmeric with Muscarinic Acetylcholine receptor M2 and PTGS2; I: The virtual docking of palmitic acid and PTGS2. J represents the virtual docking of demethoxycurcumin and PTGS2.

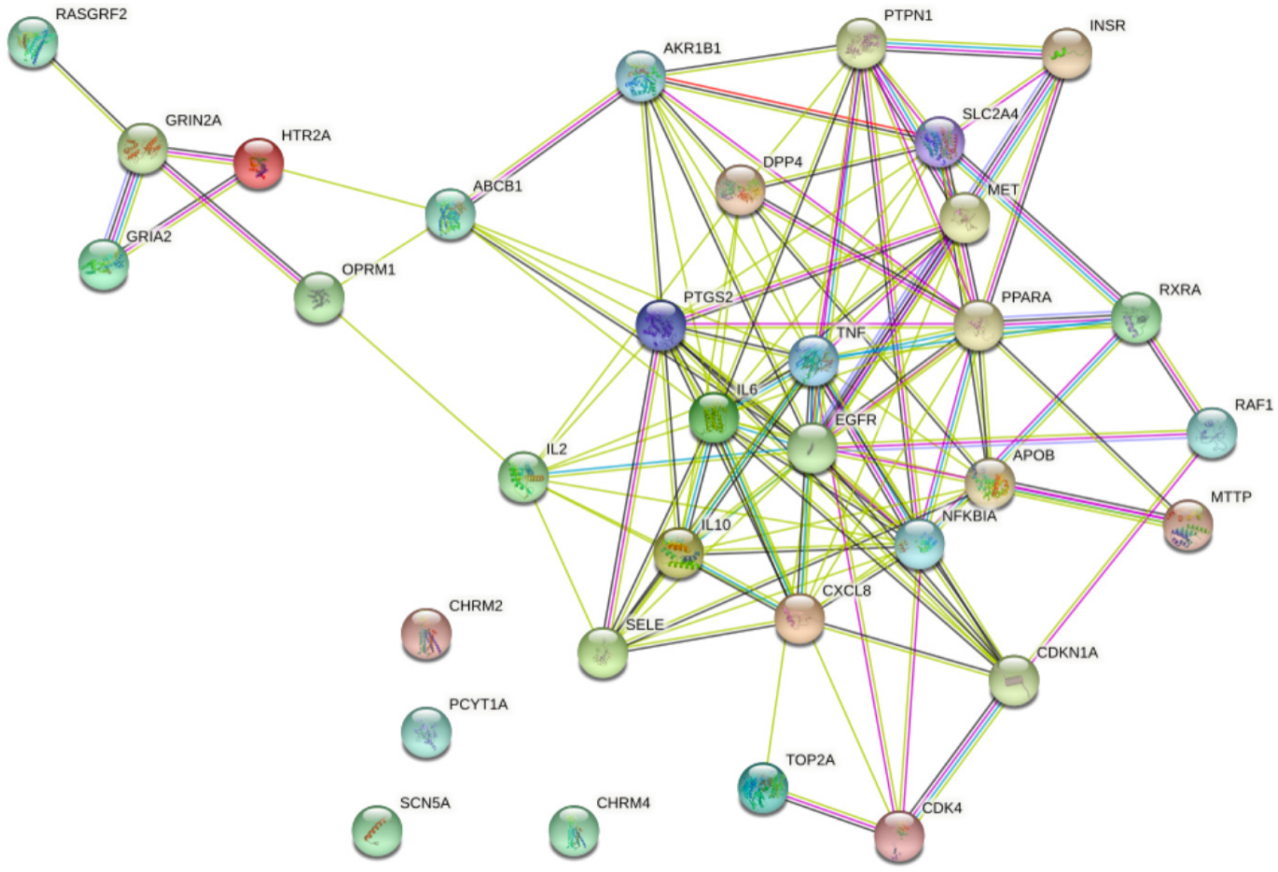

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Figure 6 Interaction diagram of target proteins. The edges with different colors represent different association relationships. Among the 36 targets, except for Muscarinic Acetylcholine receptor M2 (CHRM2), CHRM4, Choline-phosphate cytidylyltransferase A and Sodium channel protein type 5 subunit alpha, the remaining 32 target proteins are closely related to each other and may participate in multiple pathways.

neuroactivated-receptor interactions, and the FoxO signaling pathway, the network topological properties of the human T-cell leukemia virus-infection signaling pathway were also found to be prominent in the RHP network pharmacology analysis; however, there is no specific research result on

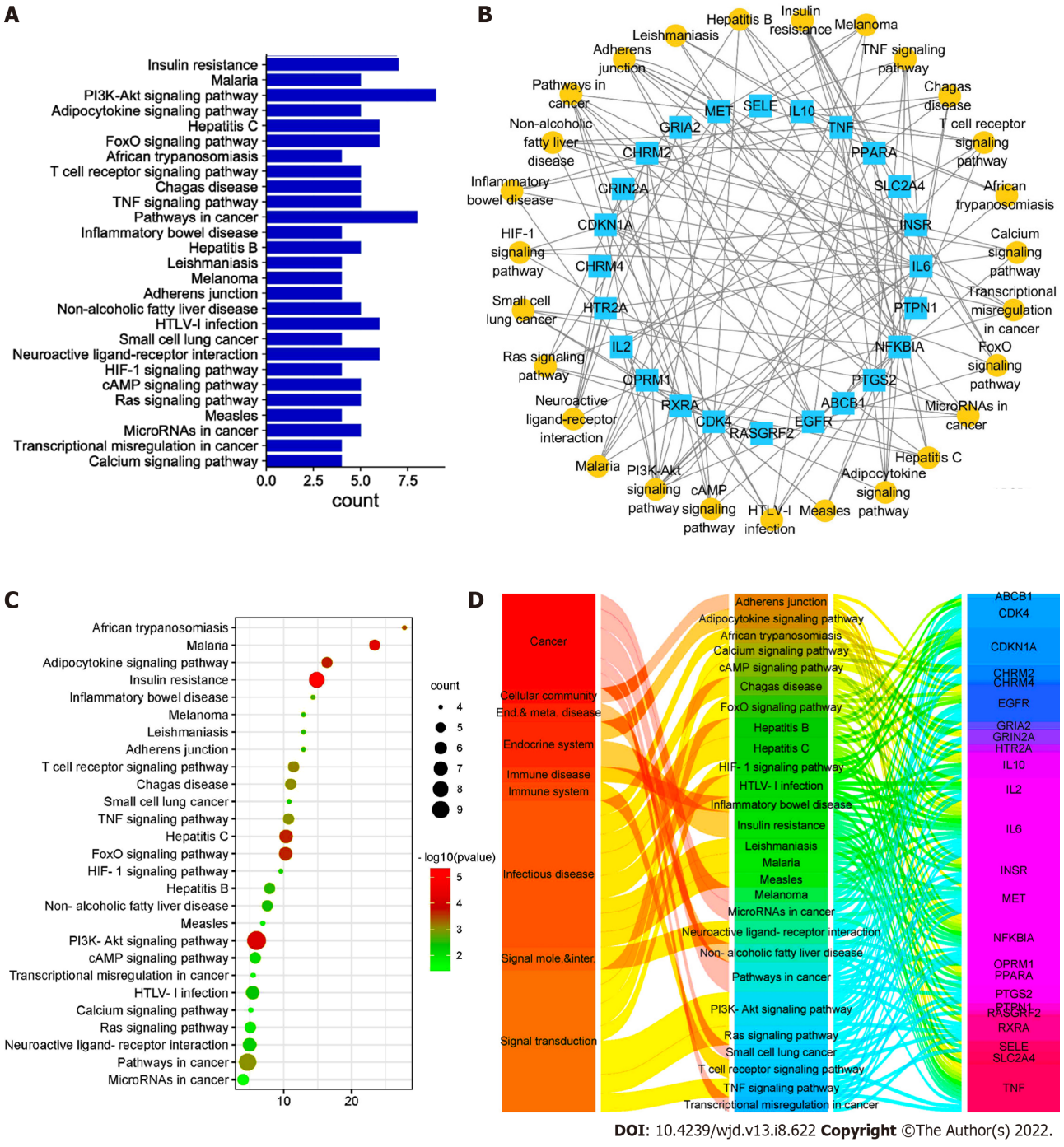

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Figure 7 Target-pathway diagram of Ruyi Jinhuang powder-diabetic foot ulcer. A: A bar graph of Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway enrichment. The left side is the pathway name, and the right side is the number of targets contained; B: The target-path network relationship diagram. The yellow circle represents 27 pathways, and the blue square represents 36 targets; C: An enriched bubble diagram of the KEGG signaling pathway. The left side is the pathway name, and the abscissa is the fold enrichment. The bubble size corresponds to the number of targets contained, and the color category corresponds to the *P* value; D: The correspondence diagram of pathway categories. The first column is the ownership of the pathway, the second column is the pathway, and the third column is the target.

how this pathway affects DFU and needs further study.

CONCLUSION

RHP, a traditional Chinese medicine formula, may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses, and regulating cell proliferation, differentiation and apoptosis through other specific mechanisms. The key active components were luteolin, methyl palmitate, ar-

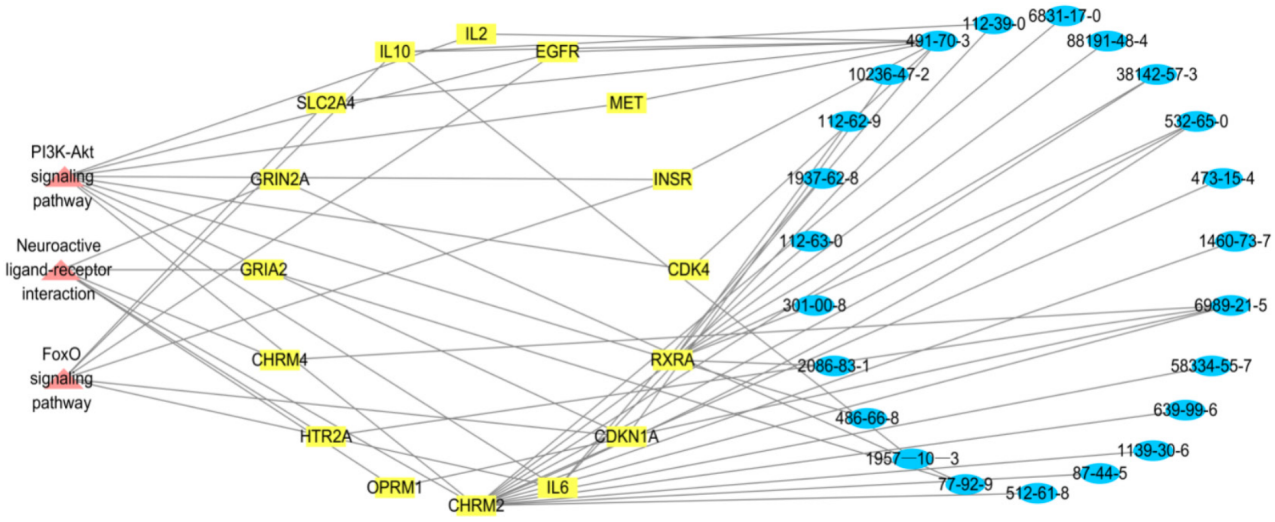

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Figure 8 Critical pathway-target-component network relationship diagram of Ruyi Jinhuang powder. Pink represents the pathway, yellow represents the target and blue represents the components. It was shown that Muscarinic Acetylcholine receptor M2 and Retinoid X Receptor alpha are common targets of the three pathways. Luteolin, atractylenone and arylodone are the key components in the three pathways.

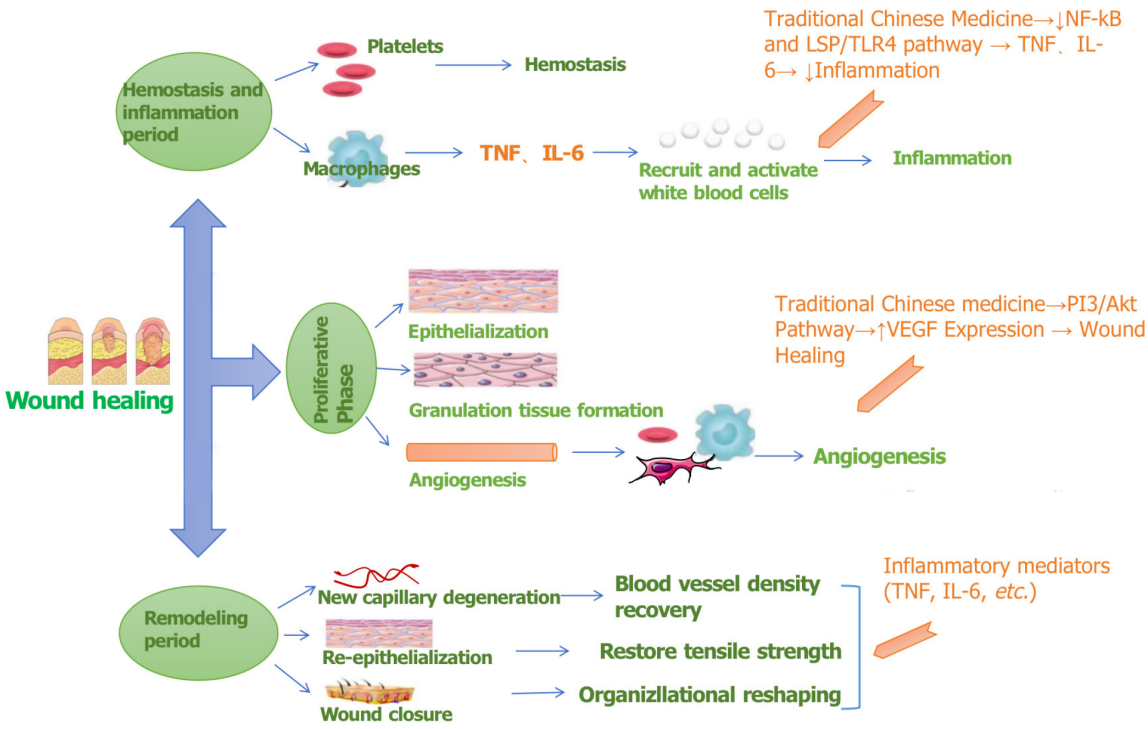

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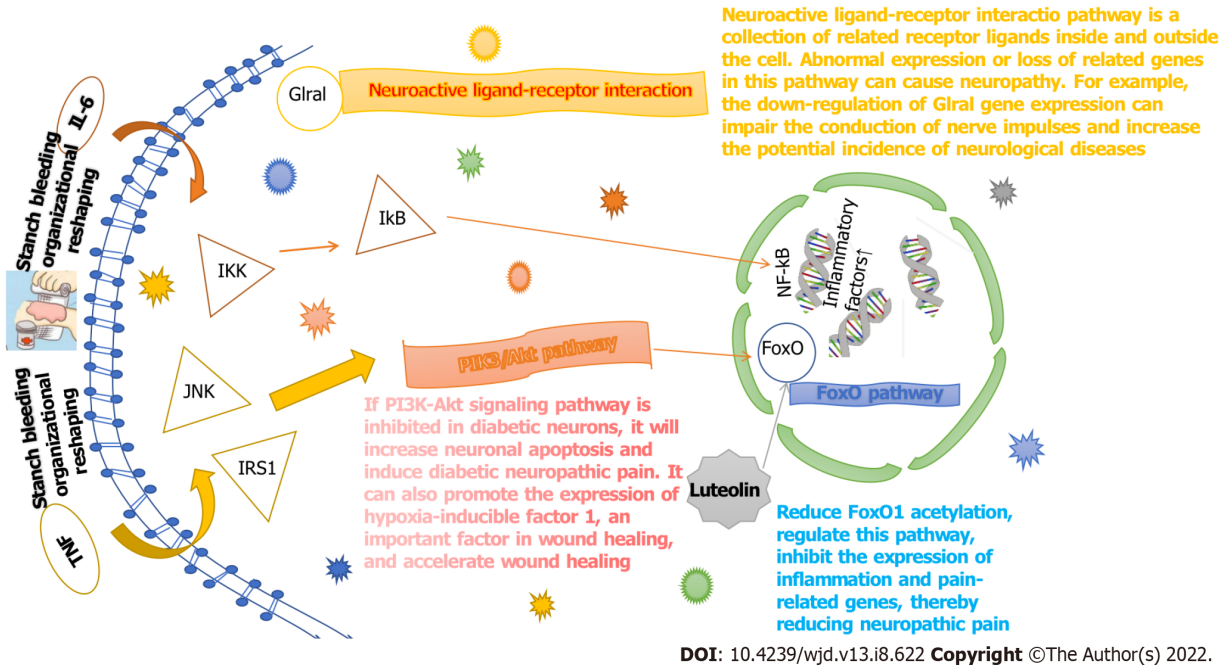
Figure 9 Wound healing mechanism diagram. TNF: Tumor necrosis factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; NF-kB: Nuclear factor-kappaB.

turmeric, methyl linoleate, palmitic acid, demethoxycurcumin, and naringin.



DOI: 10.4239/wjd.v13.i8.622

Figure 10 Target pathway mechanism of Ruyi Jinhuang powder. TNF: Tumor necrosis factor; IL: Interleukin; NF-kB: Nuclear factor-kappaB; IKK: IkappaB kinase; JNK: c-Jun N-terminal kinase; IkB: Inhibitor kB; IRS1: Insulin receptor substrate-1.

ARTICLE HIGHLIGHTS

Research background

Diabetic foot ulcer (DFU) seriously affects the quality of life of patients. Traditional Chinese medicine has a unique effect in the treatment of skin ulcerative diseases. Ruyi Jinhuang powder (RHP) is one of the classic prescriptions in traditional Chinese medicine and is widely used in clinical practice.

Research motivation

Although there have been studies suggesting that RHP has a therapeutic effect on DFU, there is a lack of research that further verify the mechanism of RHP to promote wound healing.

Research objectives

The effective components of RHP were extracted and identified by chromatography-mass spectrometry, and the obtained chemical components were analyzed by network pharmacology methods to predict its therapeutic mechanism. Gas chromatography–mass spectrometry (MS) and ultra-performance liquid chromatography-MS were used to separately identify the chemical constituents.

Research methods

Sprague Dawley rats were injected with streptozotocin to establish the DFU model. Hematoxylin-eosin staining was used to observe the wound tissue under an electron microscope. Medicine Systems Pharmacology database to obtain the target information, and the molecular docking of important components and key targets was performed in Discovery Studio software. Cytoscape software was used to visualize and analyze the relationship between the chemical composition, targets and Traditional Chinese Medicine network.

Research results

RHP was shown to promote the healing of diabetic foot ulcers in rats by affecting fibroblasts and nerve cells. A total of 89 chemical components were obtained by chromatography-mass spectrometry. Network pharmacological analysis revealed that RHP was associated with 36 targets and 27 pathways in the treatment of DFU.

Research conclusions

Our results indicated that RHP may play a role in the treatment of DFU through these target pathways by affecting insulin resistance, altering the nervous system and immune system, participating in inflammatory responses and regulating cell proliferation, differentiation and apoptosis and through other specific mechanisms.

Research perspectives

We found that RHP plays a role in the treatment of diabetic foot ulcers through multiple targets and channels. However, the way in which specific components are combined with target proteins needs to be further explored.

FOOTNOTES

Author contributions: Li XY, Zhang XT, Jiao YC, Chi H, Xiong TT and Zhang WJ, Li MN performed the experiments and acquired and analyzed the data; Li XY, Zhang XT and Wang YH wrote the manuscript; all authors approved the final version of the article.

Supported by National Natural Science Foundation of China, No. 82074025; Scientific Research Project of Heilongjiang Health Committee, No. 2020-293; and Scientific and Technological Innovation Project for College Students of Heilongjiang University of Chinese Medicine, No. 2021-13.

Institutional review board statement: This study does not involve human.

Institutional animal care and use committee statement: This study was approved by Ethics Committee of Heilongjiang University of Chinese Medicine, Harbin, China.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Fan JR
L-Editor: A
P-Editor: Chen YX

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 643-653

DOI: 10.4239/wjd.v13.i8.643

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Case Control Study

Association of rs1137101 with hypertension and type 2 diabetes mellitus of Mongolian and Han Chinese

Ke-Yu Zhao, Meng-Lu Yuan, Yun-Na Wu, Hong-Wei Cui, Wen-Yan Han, Jing Wang, Xiu-Lan Su

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Javor E, Croatia; Mahmoud MZ, Saudi Arabia; Mrzljak A, Croatia

Received: February 17, 2022
Peer-review started: February 17, 2022
First decision: May 30, 2022
Revised: June 14, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: August 15, 2022

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Abstract

BACKGROUND

Hypertension (HTN) and type 2 diabetes mellitus (T2DM) are often coincident, and each condition is considered a risk factor for the other. Both occur frequently in the Inner Mongolia region of China. The reasons for differences in risk between Han and Mongolian ethnic groups are not known. The *LEPR* gene and its polymorphism, rs1137101 (Gln223Arg), are both considered risk factors for HTN and T2DM, but any role of rs1137101 in the occurrence of HTN + T2DM remains unclear for Mongolian and Han populations in the Inner Mongolia region.

AIM

To investigate the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia.

METHODS
A total of 2652 subjects of Han and Mongolian ethnic origins were enrolled in the current study, including 908 healthy controls, 1061 HTN patients and 683 HTN patients with T2DM.

RESULTS
The association between the rs1137101 polymorphism and HTN with T2DM was analyzed, and differences between Han and Mongolian individuals assessed. There was a significant correlation between rs1137101 and HTN (co-dominant, dominant, over-dominant and log-additive models) and HTN + T2DM (co-dominant, dominant, over-dominant and log-additive models) after adjustment for sex and age in individuals of Mongolian origin. rs1137101 was significantly associated with HTN (co-dominant, recessive and log-additive models) and HTN + T2DM (co-dominant, dominant, over-dominant and log-additive models) in the Han Chinese population.

CONCLUSION
Mongolian and Han subjects from Inner Mongolia with HTN who had rs1137101 were protected against the development of T2DM. Allele A has the opposite impact on the occurrence of HTN in Mongolian and Han Chinese populations.

Key Words: rs1137101; Mongolian; Han Chinese; Hypertension; Type 2 diabetes mellitus; Associate study

Core Tip: Hypertension and type 2 diabetes mellitus are often coincident, and each condition is a risk factor for the other. It is unknown why there are differences in risk between Han and Mongolian ethnic groups. The *LEPR* gene and its polymorphism, rs1137101 (Gln223Arg), are considered risk factors for the occurrence of hypertension and type 2 diabetes mellitus. The current study investigated the relationship between rs1137101 and the occurrence of hypertension with type 2 diabetes mellitus in Mongolian and Han populations in Inner Mongolia. Differences between the two populations were analyzed. The aim was to inform further research on advanced metabolic disease.

Citation: Zhao KY, Yuan ML, Wu YN, Cui HW, Han WY, Wang J, Su XL. Association of rs1137101 with hypertension and type 2 diabetes mellitus of Mongolian and Han Chinese. *World J Diabetes* 2022; 13(8): 643-653
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/643.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.643

INTRODUCTION

The causes of hypertension (HTN) are multifactorial, and the condition is in turn a risk factor for cardiovascular disease and nephropathy[1]. Current estimates put a global figure of 1.3 billion[2,3] on the number of people with high blood pressure, an estimate that is set to rise to 1.6 billion by 2025[2,4]. Advanced age, gender, obesity and genotype are all risk factors for HTN[2]. Diabetes mellitus (DM) is another public health problem that has increased rapidly over recent years with 80%-90% patients having type 2 DM (T2DM)[5,6]. Epidemiological studies have shown that HTN is a major risk factor for T2DM[7]. One-third of HTN patients also have T2DM and are at an increased risk of cardiovascular disease and mortality[8,9].

The leptin (LEP) receptor (LEPR) is a transmembrane protein encoded by the *LEPR* gene. Several variants have been characterized, and there is widespread expression throughout the body's tissues[10]. The LEP hormone is known to have roles in the regulation of hunger, energy balance, metabolism, reproduction and insulin secretion mediated by binding to LEPR[11,12]. Binding of LEP to its hypothalamic receptor has been shown to raise blood pressure in mice, and blockade of LEPR resulted in lower values[13,14]. LEPR has roles in insulin secretion, and its activity is relevant to the development of insulin resistance[12,15]. Indeed, a recent study has correlated *LEPR* polymorphisms with DM and HTN[16,17]. Among the Han Chinese population, the *LEPR* gene polymorphism, rs13306519, has been associated with DM and rs12037879 with HTN[5]. Moreover, rs1137100 (Arg109Lys) and rs8179183 (Lys656Asn) have been associated with both DM and HTN[15,18].

The *LEPR* gene polymorphism, rs1137101, is located on chromosome 1p31 and involves a substitution of the 223rd amino acid residue, gln (Q) for Arg (R). This mutation affects the ObRIg domain, according to the PFAM database (http://pfam.xfam.org/protein/P48357; Figure 1A and Table 1). Construction of a 3D model of the region including amino acids 126 to 533 using Swiss-model software (https://swissmodel.expasy.org/) revealed a consequent change in protein structure (Figure 1B). These

Table 1 Domain boundaries and score for each of the domains

Source	Domain	Start	End	Gathering threshold (bits)		Score (bits)		E-value	
				Sequence	Domain	Sequence	Domain	Sequence	Domain
Pfam	ObR_Ig	126	233	25.8	25.8	170.5	61.1	3.50E-47	3.9E-13
Pfam	Lep_receptor_Ig	329	420	28.8	28.8	84.8	84.8	1.00E-20	1.00E-20
Pfam	ObR_Ig	431	533	25.8	25.8	170.5	112.7	3.50E-47	3.40E-29
Transmembrane	NA	840	862	NA	NA	NA	NA	NA	NA
Low_complexity	NA	849	863	NA	NA	NA	NA	NA	NA
Disorder	NA	924	927	NA	NA	NA	NA	NA	NA
Low_complexity	NA	937	946	NA	NA	NA	NA	NA	NA
Disorder	NA	966	967	NA	NA	NA	NA	NA	NA
Disorder	NA	970	973	NA	NA	NA	NA	NA	NA
Disorder	NA	975	976	NA	NA	NA	NA	NA	NA
Disorder	NA	997	1001	NA	NA	NA	NA	NA	NA
Disorder	NA	1064	1065	NA	NA	NA	NA	NA	NA

NA: No adoption.

predictions imply that the rs1137101 mutation may influence protein structure and have an impact on protein function. Previous studies have associated rs1137101 (Gln223Arg) with obesity, cancer, HTN and DM[9,19,20]. It also has been shown to be a risk factor for HTN and T2DM in the Chinese population[21,22]. The current study investigated the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia.

MATERIALS AND METHODS

Study subjects

A total of 2652 subjects, including 908 healthy controls, 1061 HTN patients and 683 patients with HTN + T2DM, were randomly selected from adult residents of Mongolia (Hohhot, Wuhai, Xilinhot) and enrolled in the study. Study participants were unrelated, and the ethnic composition was 1347 Han and 1305 Mongolian. All participants provided written informed consent. The study was performed in accordance with the declaration of Helsinki and approved by the ethical committee of the affiliated hospital of Inner Mongolia Medical University.

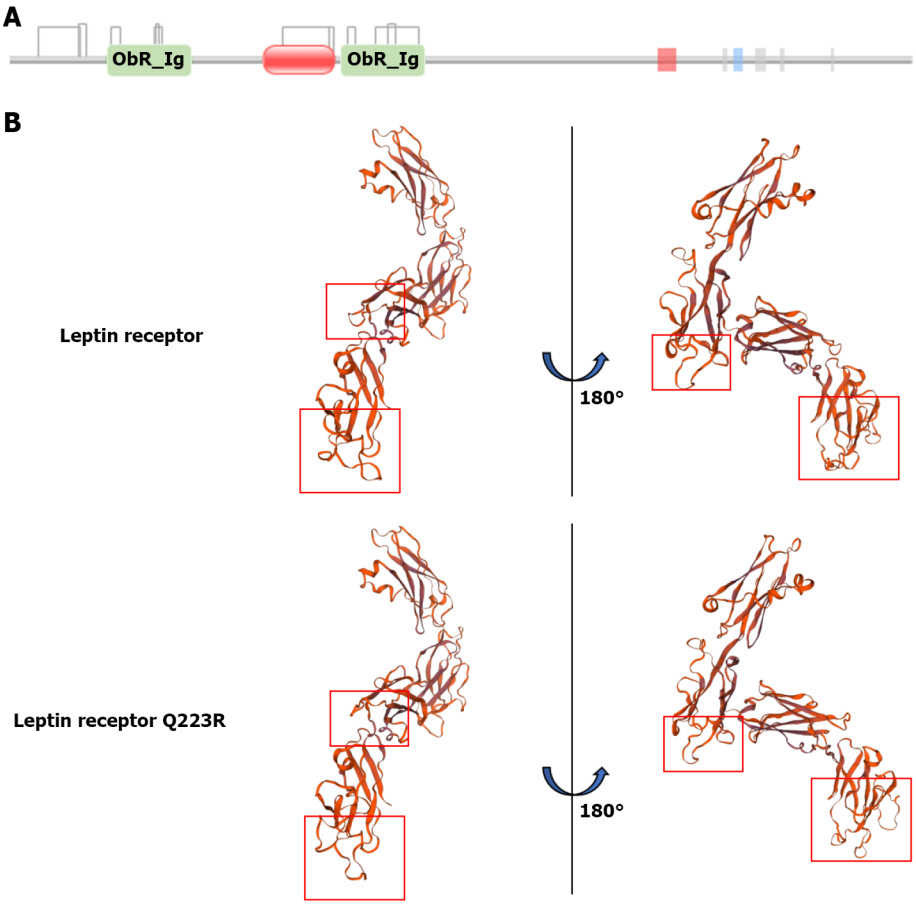
T2DM and HTN were diagnosed according to the following criteria established by the World Health Organization: HTN: Systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or current prescription for antihypertensive medication[23]. Participants with chronic renal disease, renal artery stenosis, primary hyperaldosteronism, thyroid disease, Cushing syndrome, phaeochromocytoma or other diseases known to cause HTN were excluded; T2DM: Fasting blood sugar (FBS) ≥ 7.0 mmol /L or postprandial blood glucose ≥ 11.1 mmol/L or current definitive diagnosis of T2DM[24]. Participants with T1DM, cancer or other severe metabolic disease were excluded.

Data collection

Age, weight and medical history were collected by questionnaire. Body mass index was calculated according to the formula: Mass (kg)/height^2 (m^2). Blood pressure was measured on the right arm using a mercury sphygmomanometer. Blood samples of HTN, T2DM and HTN + T2DM groups were collected after an 8 h fast. Genomic DNA was isolated from whole blood using a Maga bio plus whole blood genomic DNA purification Kit II (Hangzhou Bioer Technology co. Ltd, China) according to the manufacturer's instructions. FBS, triglyceride, cholesterol, high density lipoprotein and low density lipoprotein were measured after plasmapheresis.

Genotyping

rs1137101 (Gln223Arg) polymorphisms were assessed by PCR amplification. The primers used were forward: 5'-TTCCCCAAAAAGGCAGTTTTCA-3' and reverse: 5'-AGAAGCCACTCTTAATAC-CCCCAGT-3'. The target DNA sequences were amplified using a multiplex PCR method. Thermal



DOI: 10.4239/wjd.v13.i8.643

Figure 1 Leptin receptor domains and 3D structure. A: The PFAM database obtains the domains of the leptin receptor (LEPR) protein; B: Swiss-model was used to construct the 3D model of the leptin receptor and the leptin receptor (Q223R) protein fragment 126 to 533. The red frame represented the differences between two models.

cycling was performed for the rs1137101 loci in Gene Amp PCR system 9600 (PerkinElmer, Waltham, MA, United States) fluorescent products of ligase detection reaction differentiated by 3130xl genetic analyzer (Applied Biosystems, CA, United States).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM Corp., Armonk, NY, United States) and SNPStats (https://www.snpstats.net/start.htm)[25] software. Categorical variables were presented as frequencies. Continuous data were reported as the mean ± standard deviation. Student's *t* test was used to compare age, weight, height, body mass index, FBS, systolic blood pressure, diastolic blood pressure, triglyceride, cholesterol, high density lipoprotein and low density lipoprotein and statistical hypotheses were tested using the 2-tailed *t* test. The χ^2 test was used to analyze ethnic and gender differences. Logistic regression was used to compute the odds ratio (OR) by adjusting for age and sex and the adjusted OR is presented with 95% confidence interval. Logistic regression, Hardy Weinberg Equilibrium and five genetic models (co-dominant, dominant, recessive, over-dominant and log-additive) were calculated using SNPStats software. A value of $P < 0.05$ was considered to be significant.

RESULTS

Baseline demographic characteristics

Baseline demographic characteristics of the study population are summarized in Table 2. Significant differences were found in ethnicity, gender, age, weight, height, FBS, Systolic blood pressure, diastolic blood pressure and high density lipoprotein between cases with HTN, those with both HTN + T2DM and controls. No significant deviation from the Hardy Weinberg Equilibrium was detected (Table 3). Allele frequency was not significant in the Han population, but significant differences between Mongolian groups were observed (Table 4).

Table 2 Baseline characteristics

		Control, *n* = 908	HTN, *n* = 1061	HTN with T2DM, *n* = 683	*P* value		
					Control *vs* HTN	Control *vs* HTN + T2DM	HTN *vs* HTN + T2DM
Ethnic	Han	455	406	486	< 0.0001	< 0.0001	< 0.0001
	Mongolian	453	655	197			
Gender	Male	357	601	397	< 0.0001	< 0.0001	0.542
	Female	551	460	286			
Age		48.11 ± 15.06	54.49 ± 15.67	63.89 ± 11.17	< 0.0001	< 0.0001	< 0.0001
Weight (kg)		66.14 ± 11.06	72.32 ± 12.06	73.35 ± 12.48	< 0.0001	< 0.0001	0.2156
Height (cm)		163 ± 0.09	168 ± 0.08	161.26 ± 0.10	< 0.0001	0.6110	0.0032
BMI (kg/m^2)		25.27 ± 8.50	25.56 ± 3.63	26.48 ± 5.12	0.4067	0.7588	0.8723
FBS (mmol/L)		5.06 ± 0.49	5.73 ± 0.77	8.59 ± 3.37	0.0721	< 0.0001	< 0.0001
SBP (mm Hg)		117.21 ± 14.27	151.10 ± 18.94	166.47 ± 17.53	< 0.0001	< 0.0001	< 0.0001
DBP (mm Hg)		77.20 ± 7.95	88.59 ± 12.74	100.84 ± 13.31	< 0.0001	< 0.0001	< 0.0001
TG (mmol/L)		1.63 ± 1.06	2.24 ± 1.52	2.59 ± 12.14	0.0626	0.0051	0.4768
CHO (mmol/L)		4.53 ± 1.30	4.47 ± 3.59	4.52 ± 1.26	0.8913	0.9984	0.8885
HDL (mmol/L)		1.44 ± 0.54	1.75 ± 0.95	1.27 ± 0.35	< 0.0001	< 0.0001	< 0.0001
LDL (mmol/L)		2.84 ± 1.00	2.87 ± 1.38	2.94 ± 11.1	0.9921	0.9225	0.9586

Data presented as mean ± SD and percentages. *P* value of < 0.05 was considered significant. BMI: Body mass index; FBS: Fasting blood sugar; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglyceride; CHO: Cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

Table 3 Hardy Weinberg equilibrium analysis

	Group	G/G	G/A	A/A	G	A	*P* value
Han, *n* = 1347	Control, *n* = 455	351	94	10	796	114	0.2
	HTN, *n* = 406	312	91	3	715	97	0.24
	HTN + T2DM, *n* = 486	394	84	8	872	100	0.21
Mongolian, *n* = 1305	Control, *n* = 453	343	101	9	787	119	0.68
	HTN, *n* = 655	436	202	17	1074	236	0.29
	HTN + T2DM, *n* = 197	151	42	4	344	50	0.53

P value of < 0.05 was considered significant. HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

LEPR gene polymorphisms and HTN in ethnic Han and Mongolian Chinese

The correlation between the *LEPR* gene polymorphism, rs1137101, and HTN in ethnic Han and Mongolian Chinese subjects was analyzed. A total of 861 subjects of Han origin (control = 455; HTN = 406) and 1108 subjects of Mongolian origin (control = 453; HTN = 655) were assessed. Logistic regression analysis was used to evaluate whether rs1137101 was independently associated with HTN after adjusting for sex and age (Table 5). Use of five inheritance models, codominant, dominant, recessive, over-dominant and log-additive, gave the following results: Co-dominant (A/G) model: OR = 0.88 (0.62-1.27); co-dominant (A/A) model: OR = 0.21 (0.05-0.80); and recessive (A/A) model: OR = 0.21 (0.05-0.82) for hypertensive Han subjects compared with controls. Results for Mongolian subjects were: Co-dominant (A/G) model: OR = 1.49 (1.12-1.97); co-dominant (A/A) model: OR = 1.47 (0.64-3.34); dominant (A/G-A/A) model: OR = 1.49 (1.13-1.95); over-dominant (A/A) model: OR = 1.47 (1.11-1.95); and log-additive model: OR = 1.40 (1.10-1.79). An association between rs1137101 and HTN was established for subjects of Mongolian ethnic origin.

Table 4 Statistics of allele and genotype frequencies

Population	Allele	All subjects count (%)	Control count (%)	HTN count (%)	HTN + T2DM count (%)	*P* value
Han, *n* = 1347	G	2383 (88)	796 (87)	715 (88)	872 (90)	0.288
	A	311 (12)	114 (13)	97 (12)	100 (10)	
	A/A	21 (2)	10 (2)	3 (1)	8 (2)	0.153
	G/A	269 (20)	94 (21)	91 (22)	84 (17)	
	G/G	1057 (78)	351 (77)	312 (77)	394 (81)	
Mongolian, *n* = 1305	G	2205 (84)	787 (87)	1074 (82)	344 (87)	0.002
	A	405 (16)	119 (13)	236 (18)	50 (13)	
	A/A	30 (2)	9 (2)	17 (3)	4 (2)	0.006
	G/A	345 (26)	101 (22)	202 (31)	42 (21)	
	G/G	930 (71)	343 (76)	436 (67)	151 (77)	

P value of < 0.05 was considered significant. HTN: Hypertension; T2DM: Type 2 diabetes mellitus.

Table 5 Association of the control and hypertension groups for Han and Mongolian Chinese

Model	Genotype	Han, *n* = 861				Mongolian, *n* = 1108			
		Control, *n* (%)	HTN, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	351 (77.1)	312 (76.8)	1	0.041	343 (75.7)	436 (66.6)	1	0.016
	A/G	94 (20.7)	91 (22.4)	0.88 (0.62-1.27)		101 (22.3)	202 (30.8)	1.49 (1.12-1.97)	
	A/A	10 (2.2)	3 (0.7)	0.21 (0.05-0.80)		9 (2.0)	17 (2.6)	1.47 (0.64-3.34)	
Dominant	G/G	351 (77.1)	312 (76.8)	1	0.23	343 (75.7)	436 (66.6)	1	0.004
	A/G-A/A	104 (22.9)	94 (23.1)	0.81 (0.57-1.15)		110 (24.3)	219 (33.4)	1.49 (1.13-1.95)	
Recessive	G/G-A/G	445 (97.8)	403 (99.3)	1	0.015	444 (98)	638 (97.4)	1	0.51
	A/A	10 (2.2)	3 (0.7)	0.21 (0.05-0.82)		9 (2.0)	17 (2.6)	1.32 (0.58-2.99)	
Over-dominant	G/G-A/A	361 (79.3)	315 (77.6)	1	0.63	352 (77.7)	453 (69.2)	1	0.0064
	A/G	94 (20.7)	91 (22.4)	0.91 (0.64-1.31)		101 (22.3)	202 (30.8)	1.47 (1.11-1.95)	
Log-additive	-	-	-	0.75 (0.55-1.04)	0.082	-	-	1.40 (1.10-1.79)	0.0059

Adjusted for sex and age. *P* value of < 0.05 was considered significant. HTN: Hypertension; OR: Odd ratio; CI: Confidence interval.

The correlation between rs1137101 and HTN with T2DM in Han and Mongolian subjects

The association of rs1137101 with HTN + T2DM was analyzed. A total of 683 subjects, composed of 197 Mongolian and 486 Han, were included. The same five genetic models, codominant, dominant, recessive, over-dominant and log-additive, were used to analyze associations between HTN + T2DM as described above for HTN. OR (adjusted for sex and age) for the five genetic models in Mongolian subjects were: Co-dominant (A/G): 0.70 (0.44-1.11); co-dominant (A/A): 1.06 (0.27-4.25); dominant (A/G-A/A): 0.72 (0.46-1.13); recessive (G/G-A/G):1.15 (0.29-4.57); over-dominant (A/G): 0.70 (0.44-1.11); and log-additive: 0.78 (0.52-1.16). OR (adjusted for sex and age) for the five genetic models in Han subjects were: Co-dominant (A/G): 0.59 (0.40-0.87); co-dominant (A/A): 0.38 (0.14-1.08); dominant (A/G-A/A): 0.56 (0.39-0.82); recessive (G/G-A/G): 0.43 (0.15-1.21); over-dominant (A/G): 0.61 (0.41-0.89); and log-additive: 0.60 (0.43-0.83). No significant differences were found in Mongolian subjects, but the genotypes GA and AA significantly decreased the risk of HTN + T2DM in Han subjects (Table 6). Thus, the LEPR polymorphism is associated with the occurrence of HTN + T2DM in Han Chinese populations but not in Mongolian Chinese.

A comparison was made between patients with HTN and those with HTN + T2DM to analyze the correlation between the *LEPR* polymorphism and the occurrence of these disorders in Mongolian and Han populations. OR (95% confidence interval) (adjusted for sex and age) for Han subjects for the same five genetic models were: Co-dominant (A/G): 0.65 (0.46-0.92); co-dominant (A/A): 1.61 (0.41-6.28); dominant (A/G-A/A): 0.68 (0.49-0.96); recessive (A/A): 1.77 (0.46-6.87); over-dominant (A/G): 0.65

Table 6 Association of rs1137101 with hypertension + type 2 diabetes mellitus (control *vs* hypertension + type 2 diabetes mellitus)

Model	Genotype	Han, *n* = 941				Mongolian, *n* = 650			
		Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	351 (77.1)	394 (81.1)	1	0.0075	343 (75.7)	151 (76.7)	1	0.3
	A/G	94 (20.7)	84 (17.3)	0.59 (0.40-0.87)		101 (22.3)	42 (21.3)	0.70 (0.44-1.11)	
	A/A	10 (2.2)	8 (1.6)	0.38 (0.14-1.08)		9 (2.0)	4 (2.0)	1.06 (0.27-4.25)	
Dominant	G/G	351 (77.1)	394 (81.1)	1	0.0024	343 (75.7)	151 (76.7)	1	0.15
	A/G-A/A	104 (22.9)	92 (18.9)	0.56 (0.39-0.82)		110 (24.3)	46 (23.4)	0.72 (0.46-1.13)	
Recessive	G/G-A/G	445 (97.8)	478 (98.3)	1	0.11	444 (98.0)	193 (98.0)	1	0.84
	A/A	10 (2.2)	8 (1.6)	0.43 (0.15-1.21)		9 (2.0)	4 (2.0)	1.15 (0.29-4.57)	
Over-dominant	G/G-A/A	361 (79.3)	402 (82.7)	1	0.01	352 (77.7)	155 (78.7)	1	0.12
	A/G	94 (20.7)	84 (17.3)	0.61 (0.41-0.89)		101 (22.3)	42 (21.3)	0.70 (0.44-1.11)	
Log-additive	-	-	-	0.60 (0.43-0.83)	0.0018	-	-	0.78 (0.52-1.16)	0.22

Adjusted for sex and age. *P* value of < 0.05 was considered significant. OR: Odd ratio; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; CI: Confidence interval.

(0.46-0.91); and log-additive: 0.75 (0.55-1.02). All values were non-significant. For Mongolian subjects, OR (adjusted for sex and age) were: Co-dominant (A/G): 0.54 (0.36-0.81); co-dominant (A/A): 0.55 (0.17-1.79); dominant (A/G-A/A): 0.54 (0.36-0.80); recessive (A/A): 0.65 (0.20-2.11); over-dominant (A/G): 0.55 (0.37-0.82); and log-additive: 0.59 (0.41-0.84). The co-dominant A/G model, dominant A/G-A/A model, over-dominant A/G model and log-additive model were all associated with a significantly decreased risk of HTN + T2DM in Mongolian and Han patients (Table 7).

DISCUSSION

HTN and T2DM are major risk factors for cardiovascular and cerebrovascular diseases, and both conditions are known to result from interactions between genetics and environment[26,27]. The *LEPR* gene has been widely studied with respect to T2DM and HTN. We have previously demonstrated an association between rs1137101 and HTN in Han subjects and an association between rs7555955 and HTN in Mongolian subjects[28]. No association was found between rs1137101 and HTN or other metabolic traits in Mexican children[29] nor with HTN or cardiovascular disease in Iranian subjects[17]. A meta-analysis did show an association between rs1137101 and T2DM[30], and a Brazilian study suggested a relationship between T2DM and being overweight[31]. Furthermore, rs1137101 was correlated with T2DM, insulin change and being overweight among the Punjabi population of North India[32]. These findings indicate that associations are very dependent on the origins of the population under study. Inner Mongolia is a vast territory with demarcation of urban, agricultural, pastoral and part-farming/part-pastoral areas. Each region has a unique lifestyle with specific eating habits, all of which have an impact on rates of HTN. Overlain on these variations are traditional risk factors, such as smoking, drinking and salt intake[33,34] plus environmental factors[35,36]. Results of the current study were not in accord with those of previous studies and discrepancies may be due to population and lifestyle differences.

The current study focused on the conditions of HTN and HTN + T2DM in ethnic Han and Mongolian populations in Inner Mongolia. There was a significant association between rs1137101 and HTN and HTN + T2DM in Han Chinese subjects. The genotypes, AA and GA, may decrease risk of HTN and HTN + T2DM for control and HTN groups. Whereas rs1137101 was associated with a significantly increased risk of HTN for control subjects, it was associated with a decreased risk of developing T2DM for HTN patients. Further investigations involving larger study populations with further data relating to environmental and lifestyle factors are required to substantiate interactions between genetics and the environment.

Table 7 Association of rs1137101 with hypertension and type 2 diabetes mellitus (hypertension and hypertension + type 2 diabetes mellitus)

Model	Genotype	Han, *n* = 892				Mongolian, *n* = 852			
		Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value	Control, *n* (%)	HTN with T2DM, *n* (%)	OR (95%CI)	*P* value
Co-dominant	G/G	312 (76.8)	394 (81.1)	1	0.034	436 (66.6)	151 (76.7)	1	0.0067
	A/G	91 (22.4)	84 (17.3)	0.65 (0.46-0.92)		202 (30.8)	42 (21.3)	0.54 (0.36-0.81)	
	A/A	3 (0.7)	8 (1.6)	1.61 (0.41-6.28)		17 (2.6)	4 (2.0)	0.55 (0.17-1.79)	
Dominant	G/G	312 (76.8)	394 (81.1)	1	0.026	436 (66.6)	151 (76.7)	1	0.0016
	A/G-A/A	94 (23.1)	92 (18.9)	0.68 (0.49-0.96)		219 (33.4)	46 (23.4)	0.54 (0.36-0.80)	
Recessive	G/G-A/G	403 (99.3)	478 (98.3)	1	0.39	638 (97.4)	193 (98.0)	1	0.46
	A/A	3 (0.7)	8 (1.6)	1.77 (0.46-6.87)		17 (2.6)	4 (2.0)	0.65 (0.20-2.11)	
Over-dominant	G/G-A/A	315 (77.6)	402 (82.7)	1	0.012	453 (69.2)	155 (78.7)	1	0.0028
	A/G	91 (22.4)	84 (17.3)	0.65 (0.46-0.91)		202 (30.8)	42 (21.3)	0.55 (0.37-0.82)	
Log-additive	-	-	-	0.75 (0.55-1.02)	0.067	-	-	0.59 (0.41-0.84)	0.0024

Adjusted for sex and age. *P* value of < 0.05 was considered significant. OR: Odd ratio; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; CI: Confidence interval.

CONCLUSION

The current study investigated the impact of the polymorphism rs1137101 on HTN in Mongolian subjects. Mongolian and Han subjects with HTN who had rs1137101 were protected against the development of T2DM, and rs1137101 decreased the risk of HTN and HTN + T2DM for the Han Chinese population of Inner Mongolia. In contrast with its protective role in the Han population, rs1137101 increased the risk of HTN for the Mongolian population.

ARTICLE HIGHLIGHTS

Research background

Hypertension (HTN) and type 2 diabetes mellitus (T2DM) are each considered a risk factor for the other. Both occur frequently in the Inner Mongolia region of China. rs1137101 is a potential risk factor for the occurrence of HTN and T2DM but the association between rs1137101 and HTN + T2DM in the Mongolian and Han population in Inner Mongolia remains unknown.

Research motivation

The association between rs1137101 and occurrence of HTN + T2DM has not been fully elucidated for Mongolian and Han populations in the Inner Mongolia region.

Research objectives

To investigate the relationship between rs1137101 and the occurrence of HTN with T2DM in Mongolian and Han populations in Inner Mongolia. To illuminate the association between the rs1137101 polymorphism and HTN with T2DM by analyzing differences between Han and Mongolian Chinese.

Research methods

Data relating to blood samples, blood pressure, weight, height and other body indices among Chinese populations in Inner Mongolia. The rs1137101 polymorphism was measured. Data was analyzed by SPSS 22.0 and SNPstats software (https://www.snpstats.net/start.htm) to correlate rs1137101 with HTN + T2DM in Mongolian and Han populations in Inner Mongolia.

Research results

The association between the rs1137101 polymorphism and HTN with T2DM was analyzed, and differences between Han and Mongolian individuals were assessed. There was a significant correlation between rs1137101 with both HTN after adjustment for sex and age in individuals of Mongolian origin. rs1137101 was significantly associated with HTN and HTN + T2DM in the Han Chinese population.

Research conclusions

There was significant correlation between rs1137101 and control and HTN/HTN + T2DM in Han and Mongolian subjects. Mongolian and Han subjects with HTN who had rs1137101 were protected against the development of T2DM. rs1137101 decreased the risk of HTN and HTN + T2DM for the Han Chinese population of Inner Mongolia. By contrast, rs1137101 increased the risk of HTN for the Mongolian population.

Research perspectives

The current study analyzed the association between rs1137101 and HTN/HTN + T2DM by comparing control, HTN and HTN + T2DM groups and found rs1137101 to be associated with HTN and HTN + T2DM in Mongolian and Han populations in Inner Mongolia. Further investigations involving larger study populations with further data relating to environmental and lifestyle factors are required to substantiate interactions between genetics and the environment.

FOOTNOTES

Author contributions: Zhao KY, Yuan ML, Wu YN, Cui HW, Han WY, Wang J and Su XL designed the research study; Zhao KY, Wang J, Yuan ML and Su XL performed the research; Su XL and Zhao KY contributed new reagents and analytic tools; Zhao KY, Yuan ML and Su XL analyzed the data and wrote the manuscript; All authors have read and approved the final manuscript.

Supported by National Natural Science Foundation of China, No. 81260058.

Institutional review board statement: The study was reviewed and approved by the Affiliated Hospital of Inner Mongolia Medical University Institutional Review Board, No. 2015003.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 654-664

DOI: 10.4239/wjd.v13.i8.654

ISSN 1948-9358 (online)

SYSTEMATIC REVIEWS

Metformin toxicity: A meta-summary of case reports

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gheshlaghi F, Iran; Neagu TP, Romania; Shen F, China; Swai J, Canada

Received: March 22, 2022
Peer-review started: March 22, 2022
First decision: May 11, 2022
Revised: May 17, 2022
Accepted: July 25, 2022
Article in press: July 25, 2022
Published online: August 15, 2022

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Abstract

BACKGROUND

Metformin is arguably the most commonly prescribed oral hypoglycemic agent for the management of diabetes. Due to the lack of randomized control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of patients with metformin induced toxicity has come from case reports or series.

AIM

To analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity by reviewing the published case reports and series.

METHODS

We performed a systematic search from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases using the terms "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were: (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin in adults, published in the English language. Data regarding baseline demographics, clinical presentation, therapeutic interventions, intensive care unit course and overall outcome were collected.

RESULTS

Two hundred forty-two individual cases were analysed, from 158 case reports and 26 case series, with a cumulative mortality of 19.8%. 214 (88.4%) patients were diabetics on metformin. 57 (23.6%) had acute ingestion, but a great majority (76.4%) were on metformin in therapeutic doses when they developed toxicity.

Metformin associated lactic acidosis (MALA) was the most commonly reported adverse effect present in 224 (92.6%) patients. Most of the patients presented with gastrointestinal and neurological symptoms and a significant number of patients had severe metabolic acidosis and hyperlactatemia. The organ support used was renal replacement therapy (RRT) (68.6%), vasopressors (58.7%) and invasive mechanical ventilation (52.9%). A majority of patients (68.6%) received RRT for toxin removal, renal dysfunction and correction of MALA. Patients with lowest pH and highest serum lactate and metformin levels also had favourable outcomes with use of RRT.

CONCLUSION

Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute deterioration in renal functions. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. Despite severe MALA and the need for multiple organ support, they may have good outcomes, especially when RRT is used. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

Key Words: Extracorporeal toxin removal; Haemodialysis; Metformin associated lactic acidosis; Metformin overdose; Renal replacement therapy

Core Tip: Metformin may be associated with significant toxicity, even when used in therapeutic doses, of which metformin associated lactic acidosis is the most commonly reported toxicity. These patients may have favourable outcomes in spite of consumption of high doses, severe acidosis, and high serum lactate and metformin concentrations. Early aggressive supportive care, use of renal replacement therapy for toxin removal and organ support may help in improving outcomes.

Citation: Juneja D, Nasa P, Jain R. Metformin toxicity: A meta-summary of case reports. *World J Diabetes* 2022; 13(8): 654-664
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/654.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.654

INTRODUCTION

Metformin is arguably the most commonly prescribed oral hypoglycemic agent (OHA) for the management of diabetes mellitus (DM). In addition to its hypoglycemic properties, it has the potential to reduce micro and macro vascular complications associated with DM and have a clinically beneficial role in reducing serum lipids levels and body weight[1,2]. Use of metformin in the management of type II DM has been shown to reduce all-cause mortality and risk of cardiovascular complications[3].

The primary mode of action of metformin is to reduce hepatic glucose production. In addition, it also exerts hypoglycemic effect through the neuroendocrine axis, enhancing cellular uptake of glucose and reducing insulin resistance[4]. It is considered a very safe drug and is generally not associated with hypoglycemia. Rarely patients may develop toxicity related to its use. Metformin associated lactic acidosis (MALA) has been defined as serum lactate levels above 5 mmol/L and arterial pH below 7.35 in association with metformin exposure[5]. It is a rare complication with a reported incidence of 1-30 cases *per* 100000 patient years but is associated with a high mortality rate of 25%-50%[6,7].

Despite severe acidosis, patients with MALA may have good clinical outcomes if it is recognised early and aggressive resuscitative measures are initiated[8]. In addition, certain therapeutic interventions like extracorporeal toxin removal (ECTR), if instituted timely, may improve survival in select patient subgroups and hence, it is currently recommended in patients with severe metformin toxicity[9]. Even though MALA remains the most dreaded complication associated with metformin use, other complications have also been reported that may require hospitalisation. Due to the lack of randomised control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of these patients have come from case reports and case series. Hence, we conducted this scoping review of case reports and series to analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity requiring hospitalisation and acute intervention.

MATERIALS AND METHODS

We performed a systematic search for this review from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases from January 1, 1975 till December 31, 2021. The search terms used were "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were: (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin. Further, it was filtered for the literature published in the English language and on adult (> 18 years) humans. We excluded: (1) Conference abstracts; and (2) Case reports or series which did not have individual biochemical data. The authors screened all the search results to include only the relevant literature for metformin toxicity. Duplicate articles from different search databases were excluded.

All the case reports and case series were evaluated, and the data were extracted for patient demographics, clinical symptomatology, clinical interventions including extracorporeal therapies (ECT), intensive care unit (ICU) course, need for organ support and outcomes. Concomitant use of nephrotoxic drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), angiotensin-converting-enzyme inhibitors (ACE-Is), angiotensin II receptor blockers (ARBs), aminoglycoside antibiotics and diuretics was also made. A datasheet for evaluation was further prepared.

Statistical analysis

The prepared datasheet was evaluated by Excel, Microsoft office 2019. Categorical variables were presented as frequency and percentage. Median (interquartile range) or mean ± SD was used for continuous variables. Qualitative correlation statistics were analysed by Chi-square test and Fisher's exact test. A P value of < 0.05 was deemed significant. Unless otherwise indicated, all the statistical analyses were done using SPSS (version 25.0, IBM SPSS Inc., Chicago, IL, United States). Tabulation and final documentation were done using MS Office software (MS office 2019, Microsoft Corp, WA, United States).

RESULTS

This review was performed using PRISMA 2009 checklist (Figure 1). Finally, 184 studies with 242 unique patients that met all the inclusion criteria were included (Supplementary material). Most of the included patients were from the United States of America (66, 27.3%) and the United Kingdom (30, 12.4%) (Figure 2). 185 (76.4%) patients developed toxicity while on chronic therapeutic doses of metformin while 57 (23.6%) patients developed toxicity after an acute overdose of the drug.

The commonly reported symptoms were gastrointestinal (vomiting 52.5%, abdominal pain 40%) and neurological (altered mental status 36%, loss of consciousness 11.6%). Two hundred fourteen (88.4%) patients had underlying diabetes and were on metformin (Table 1).

MALA was the most commonly reported adverse effect in 224 (92.6%) patients. Other reported isolated adverse effects were encephalopathy in 6 (2.5%) patients, hepatitis or acute liver failure in 5 (2.1%) patients, hypoglycemia in 4 (1.6%) patients, and psychosis, vitamin B12 deficiency and acute pancreatitis in 1 (0.4%) patient each.

Overall, 57 (23.6%) patients had acute ingestion, out of which 52 were suicidal, three were reported as accidental and in two cases the cause was not reported or was unclear. The reported median dose consumed by these patients was 42.5 gms (interquartile range 24.8–61.5 gms). Out of these 57 cases, there were 11 deaths with a cumulative mortality rate of 19.3%. Sixteen patients with acute intoxications had a history of co-ingestion of other drugs.

The mean duration of presentation after acute intoxication was 10.9 (± 13.8) hours. Activated charcoal (6.6%) and gastric lavage (5.8%) was rarely employed to reduce the absorption of metformin in patients with acute intoxication. Intravenous soda-bicarbonate was most commonly used among the other therapies employed in 65.3% of patients.

Arterial blood gas at presentation was available in 214 (88.4%) patients and serum metformin concentration was measured only in 58 (24%) cases (Table 2).

Overall, 185 (76.4%) patients were on long-term therapeutic doses of metformin when they developed metformin toxicity. These patients were on metformin doses ranging from 250–3000 mg/day (median 1625 mg/day). The cumulative mortality was 37/185 (20%) in this group of patients. Out of these 185 patients, 38 patients had underlying chronic kidney disease (CKD) and 73 patients had documented reasons, which may have caused acute renal dysfunction precipitating metformin toxicity. These reasons included acute gastroenteritis leading to dehydration (36 patients), ACE-I inhibitors (22 patients), NSAIDs (18 patients), diuretics (16 patients), ARBs (8 patients), IV contrast (7 patients), post-operative (5 patients), acute urinary tract infection (5 patients), anti-retroviral drugs like tenofovir (4 patients), aminoglycosides (2 patients), and obstructive uropathy (1 patient). Several patients had multiple risk factors for acute kidney injury.

Renal dysfunction was the most common organ dysfunction (74%), followed by cardiac (59.5%) and pulmonary (47.1%). One hundred sixty-six (68.6%) patients underwent renal replacement therapy (RRT)

Table 1 The commonly reported symptoms (mean ± SD)

Parameter	Number of patients (*n* = 242)
Age	59.3 (16) yr
Gender, *n* (%)	Females, 126 (52.1)
	Males, 115 (47.5)
	Not mentioned, 1 (0.4)
Clinical presentation, *n* (%)	Vomiting, 127 (52.5)
	Abdominal pain, 96 (40)
	Altered mental status, 87 (36)
	Shock, 43 (17.8)
	Breathlessness, 41 (16.9)
	Loss of consciousness, 28 (11.6)
	Anuria, 22 (8.3)
	Cardiac arrest, 5 (2)
	Others, 15 (6.2)
Comorbidities, *n* (%)	Diabetes, 214 (88.4)
	Hypertension, 94 (38.8)
	Coronary artery disease, 34 (14.1)
	Chronic kidney disease, 41 (16.9)
	Chronic liver disease, 6 (2.5)
	Others, 24 (9.9)
	None, 22 (9.9)
	Not mentioned, 2 (0.8)
History of psychiatric illness, *n* (%)	30 (12.4)
History of metformin use, *n* (%)	214 (88.4)
Type of ingestion, *n* (%)	Chronic use, 185 (76.4)
	Suicidal, 52 (21.5)
	Accidental, 3 (1.2)
	Unclear, 1 (0.4)
	Not mentioned, 1 (0.4)
Urine toxicology screen, *n* (%)	15 (6.2)
Time to presentation after acute intoxication (h)	10.9 ± 13.8
Hypoglycemia, *n* (%)	59 (24.4)
Therapies to reduce absorption, *n* (%)	Activated charcoal, 16 (6.6)
	Gastric lavage, 14 (5.8)
	Whole bowel irrigation, 1 (0.4)
Need for organ support, *n* (%)	RRT, 166 (68.6)
	Vasopressors, 142 (58.7)
	Invasive mechanical ventilation, 128 (52.9)
	Extracorporeal membrane oxygenation, 2 (0.8)
Type of RRT, *n* (%)	Haemodialysis, 83 (34.3)
	Continuous RRT, 60 (24.8)
	Slow low-efficiency dialysis, 13 (5.4)

	Peritoneal dialysis, 6 (2.5)
	Haem-adsorption columns, 3 (1.2)
	Plasmapheresis, 1 (0.4)
Other treatments given, *n* (%)	Sodium bicarbonate, 158 (65.3)
	Glucose/insulin, 15 (6.2)
	Methylene blue, 2 (0.8)
	ECMO, 2 (0.8)
	L-carnitine, 1 (0.4)
	High dose vitamin C, 1 (0.4)
Development of organ failure, *n* (%)	Renal, 179 (74)
	Cardiac, 144 (59.5)
	Pulmonary, 114 (47.1)
	Neurological, 88 (36.4)
	Liver, 18 (17.4)
	Haematological, 2 (0.8)
Days on RRT	3.1 ± 6.7
Days on IMV	2.2 ± 5.1
Number of sessions of RRT	2 ± 2.6
Time of initiation of RRT after presentation (h)	6.3 ± 12.7
Days in hospital	7.3 ± 11.4
Days in ICU	4.1 ± 6.6
Outcome, *n* (%)	Alive, 192 (79.3)
	Death, 48 (19.8)
	Not mentioned, 2 (0.8)

RRT: Renal replacement therapy; IMV: Invasive mechanical ventilation; ICU: Intensive care unit.

Table 2 Arterial blood gas parameters

Parameter	Mean	Standard deviation	Range
pH, at presentation	7.00	0.11	6.38-7.5
Lactates, at presentation (mmol/L)	15.7	7.6	2.1-40.2
Bicarbonate, at presentation (mmol/L)	7.7	6	1–23.7
Anion Gap, at presentation	32	10.8	10-61
Lowest pH reported	6.97	0.22	6.28–7.5
Highest lactates reported (mmol/L)	18	8.6	2.4-48
Serum metformin concentration (mcg/mL)	108.7	280	0.9-2020

for underlying renal dysfunction, metabolic acidosis correction, or metformin removal. Intermittent haemodialysis (IHD, 34.3%) was the most commonly employed method of RRT followed by continuous RRT (CRRT, 24.8%), which included both continuous veno-venous hemofiltration and continuous veno-venous hemodiafiltration. Overall, 41 (16.9%) patients had underlying CKD and were already on dialysis support. Only 17 survivors, who did not have pre-existing CKD, required RRT after hospital discharge, rest all showed complete recovery of renal function.

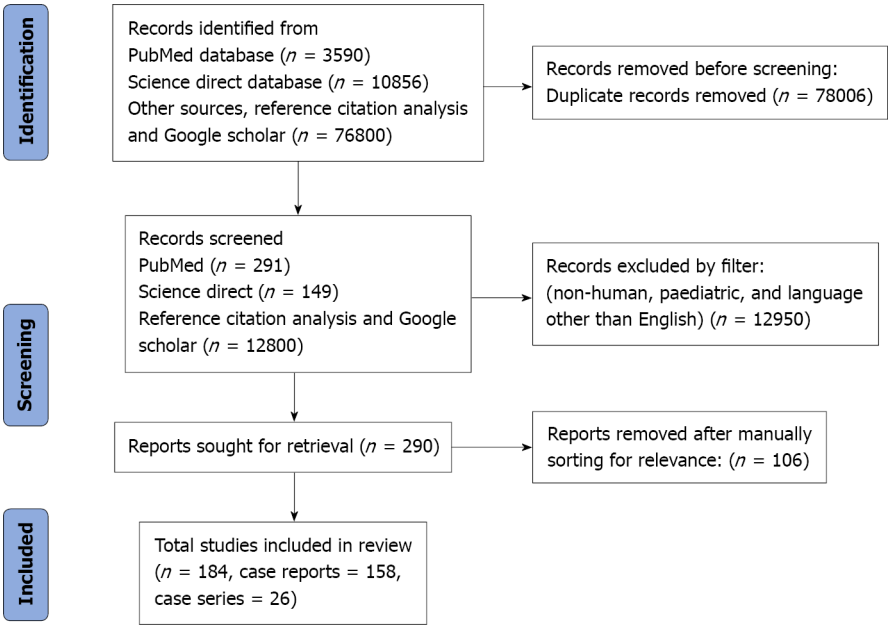

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Figure 1 PRISMA flow diagram of the selected literature for this Meta summary. The inclusion criteria were (1) Case reports or case series with individual patient details; and (2) Reported toxicity or overdose of metformin.

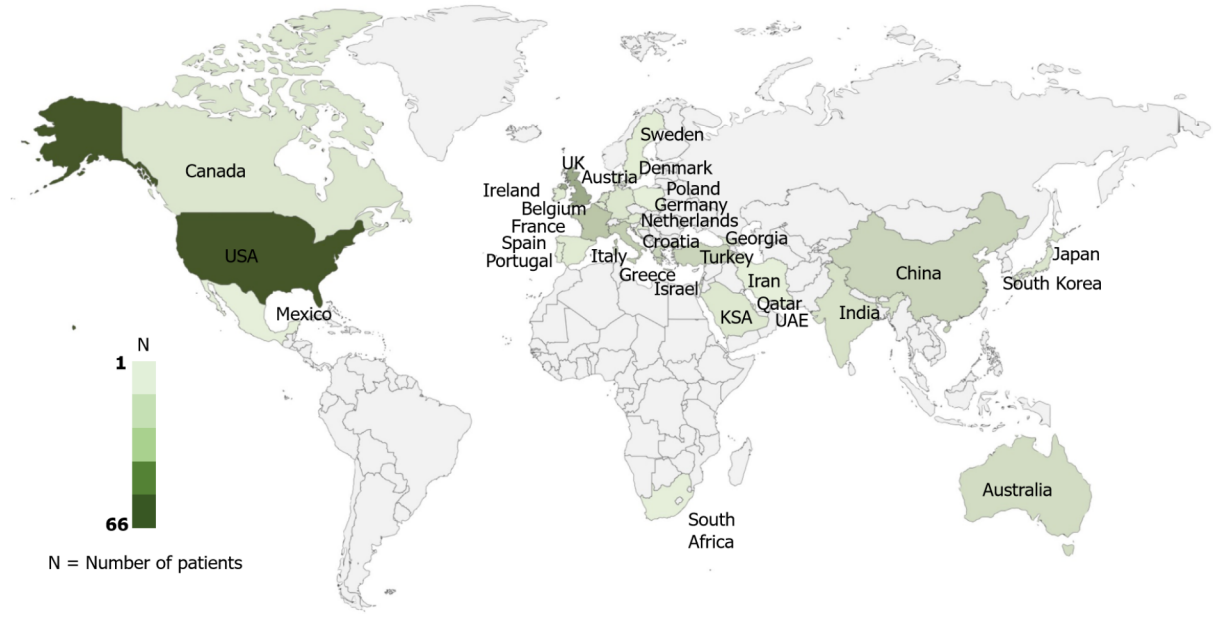

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Figure 2 Geographical distribution of the patients reported with metformin toxicity.

DISCUSSION

This review evaluated data of 242 individual patients from 158 case reports and 26 case series. Most of the patients had gastrointestinal or neurological symptoms at presentation. A great majority of patients (76.4%) developed metformin toxicity on chronic therapeutic doses. The most commonly reported side effect was MALA (92.6%). These patients had severe metabolic acidosis with hyperlactatemia and required multi organ support. RRT was employed in 68.6% of patients, and the cumulative mortality rate was 19.8%.

Several other isolated serious complications, in the absence of MALA, were also reported in many case reports. These included encephalopathy[10-14], psychosis[15], vitamin B12 deficiency[16], acute pancreatitis[17] and acute liver failure[18-22]. In our analysis, many patients (24.4%) developed hypoglycemia, attributed to sulphonylureas or other OHA that the patients were co-prescribed.

However, in a few case reports no other cause could be ascertained and hypoglycemia was attributed to metformin toxicity[23-26]. The reported incidence for development of moderate to severe hypoglycemia is 60 *per* 100000 for patients on metformin therapy, with an odds ratio of 1.42[27].

As metformin is primarily excreted by the kidneys, it is generally recommended not to use metformin in patients with underlying renal dysfunction[28]. Nonetheless, in our review we observed that 16.9% patients who developed metformin related side effects had underlying CKD. However, emerging literature supports the use of metformin in patients with mild to moderate renal dysfunction, when used in reduced dosage and regular monitoring[29].

Even though renal dysfunction is a major contributing factor for metformin toxicity, other factors like hypotension, dehydration, sepsis, ischemia, and liver impairment, which lead to increased production or impaired clearance of lactates, may also contribute to lactic acidosis. In our analysis, most of the patients on therapeutic doses of metformin who developed toxicity had some insult causing acute renal dysfunction which could have precipitated metformin toxicity. This acute insult included concomitant use of nephrotoxic drugs, acute infection, post-operative state or dehydration secondary to severe diarrhoea. Hence, it may be suggested that patients on long-term metformin should be closely monitored for the development of any side-effects in case of any acute renal insult, and concomitant use of nephrotoxic drugs should be avoided in these patients.

Patients with MALA had severe metabolic acidosis and hyperlactatemia, with the reported nadir of pH being 6.28[30]. The mainstay of therapy for MALA remains early aggressive resuscitation and organ support, as there is no specific antidote. In our analysis, intravenous soda-bicarbonate was used in a large proportion of patients (65.3%). Even though it may help in the correction of acidosis, it may lead to electrolyte imbalance and fluid overload. In addition, it does not help in the correction of the underlying cause. However, it may be reasonable to use intravenous bicarbonate in patients with severe acidemia and patients with an arterial pH less than 7.20 in the presence of underlying cardiovascular disease or hemodynamic compromise[31].

Low serum pH levels, and high lactate and metformin concentration have been associated with severe toxicity and higher mortality[32]. However, in our review, the patients with the lowest pH and highest serum lactate and metformin levels survived. All three patients with acute ingestion of massive doses of metformin (more than 100 g) survived after the institution of ECTR with complete recovery of renal functions[33-35]. In addition, patients with the highest serum metformin levels (2020 and 678 mcg/mL)[36,37] and patients with the highest presenting lactate levels (40.2 and 35.3 mmol/L)[38,39] also had favourable outcomes. The lowest pH reported was 6.28, in a post-operative diabetic CKD patient on prolonged metformin therapy, who survived after aggressive intensive care[30]. Similar findings have also been reported by other authors who found that blood pH, lactate and metformin levels were poor predictors of mortality in patients with MALA[40].

Even though metformin has a relatively large volume of distribution (1-5 L/kg), but its small molecular size (165 Da) and lack of protein binding makes it amenable for removal through ECT and use of ECTR is recommended in the management of patients with severe toxicity[9,41]. The currently recommended indications of ECTR include lactate levels above 20 mmol/L, pH less than 7.0, presence of shock, reduced consciousness or in patients with failure of standard supportive measures. The current recommendations suggest discontinuing ECTR when serum lactate levels fall below 3 mmol/L and the pH becomes more than 7.35[9].

Intermittent HD has been recommended as the RRT modality of choice for ECTR in patients with metformin toxicity as it provides rapid and superior correction of acidemia and removal of metformin and lactates[9]. Lactate clearance may also be enhanced with use of higher effluent rates and high-flux/high-efficiency dialyzers[33,42]. In addition, IHD has wider availability, lesser costs and a better safety profile. Hence, it was the most commonly used mode of ECTR as observed in our review.

CRRT may be used as the second line therapy in patients with haemodynamic instability who cannot tolerate IHD[9]. As many patients in our analysis had haemodynamic instability requiring vasopressor support, CRRT was the second most common mode of RRT, employed in 24.8% patients.

Slow low efficiency dialysis (SLED) is increasingly becoming a popular RRT option, especially in ICU patients as it can achieve rapid and efficient solute clearance while offering good haemodynamic tolerability. This fact was evidenced in our review, where SLED was used in a few patients (5.4%) for initial RRT. There are a few reports of effective use of resin or charcoal based haem-adsorbent filters in managing patients with severe metformin toxicity[43,44]. However, lack of widespread availability, higher cost, limited data regarding their efficacy and risk of complications especially haemolysis, precludes haemoperfusion (HP) using haemadsorption filters from becoming the modality of choice for ECTR. Additionally, as metformin is not protein-bound, HP and plasmapheresis do not offer any advantage over IHD. Peritoneal dialysis is rarely used for ECTR because of inefficient and slow correction of hyperlactatemia and acidosis[9].

A similar meta-summary included 253 cases and reported a cumulative mortality of 17.2% in patients with MALA[45]. The authors reported that non-survivors had significantly higher levels of lactates and metformin. Additionally, lactate levels above 20 mmol/L were significantly associated with mortality. Even though the cumulative mortality rate in our review was 19.8%, which is close to that reported by Yeh *et al*[45], our review has significant differences. The previous meta-summary had included patients only up to September 2014, so must have missed the recent changes in clinical practices which might

have happened after EXtracorporeal TReatments In Poisoning guidelines, released in 2015, recommending ECTR for metformin toxicity[9]. Yeh *et al*[45], also included conference abstracts from the EMBASE database and included publications in all languages, explaining their relatively higher case numbers[45]. On the other hand, we included only English language papers and excluded conference abstracts. In addition, we also included all patients with metformin toxicity and even those in whom ECTR was not employed.

Strength and limitations

This review compiled 184 global studies involving 242 unique patients who had developed metformin toxicity. In addition, we included only studies with individual patient's details to compare demographics, therapeutic interventions and outcomes.

The included studies were only case reports and case series without a control arm. Hence, the efficacy and cost-benefit analysis of ECTR compared to pharmacological therapy could not be performed. The studies were heterogeneous, with a high risk of bias and missing data, which may impact the generalisability of the results. As we excluded case reports or series which did not have individual biochemical data, we might have missed some relevant case reports or series.

CONCLUSION

Metformin is associated with significant toxicity, of which MALA is most commonly reported. Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute deterioration in renal function. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. However, in spite of severe lactic acidosis and need for multiple organ support they may have good outcomes, especially when RRT is used for toxin removal. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

ARTICLE HIGHLIGHTS

Research background

Metformin is arguably the most commonly prescribed oral hypoglycemic agent for the management of diabetes. Due to the lack of randomized control trials, most of the data pertaining to the clinical course, therapeutic interventions and outcomes of patients with metformin induced toxicity has come from case reports or series.

Research motivation

Despite severe acidosis, patients with metformin associated lactic acidosis (MALA) may have good clinical outcomes, if it is recognized early and aggressive resuscitation measures are initiated.

Research objectives

This study aimed to analyse the symptomology, clinical interventions and outcomes of patients presenting with severe metformin toxicity by reviewing the published case reports and series.

Research methods

We performed a systematic search from PubMed, Science Direct, *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) and Google Scholar databases using the terms "metformin" AND "toxicity" OR "overdose" OR "lactic acidosis" OR "hyperlactatemia". The inclusion criteria were case reports or case series with individual patient details; and reported toxicity or overdose of metformin in adults, published in the English language. Data regarding baseline demographics, clinical presentation, therapeutic interventions, intensive care unit course and overall outcome were collected.

Research results

Two hundred forty-two individual cases were analyzed, from 158 case reports and 26 case series, with a cumulative mortality of 19.8%. 214 (88.4%) patients were diabetics on metformin. 57 (23.6%) had acute ingestion, but 76.4% were on metformin in therapeutic doses when they developed toxicity. MALA was the most commonly reported adverse effect present in 224 (92.6%) patients. Patients with lowest pH and highest serum lactate and metformin levels also had favorable outcomes with use of renal replacement therapy.

Research conclusions

Most of the reported cases were on therapeutic doses of metformin but developed toxicity after an acute

deterioration in renal function. These patients may develop severe lactic acidosis, leading to significant morbidity and need for organ support. Despite severe MALA and the need for multiple organ support, they may have good outcomes, especially when renal replacement therapy is used. The dose of metformin, serum pH, lactate and metformin levels may indicate the severity of toxicity and the need for aggressive therapeutic measures but may not necessarily indicate poor outcomes.

Research perspectives

Larger trials may be required to identify the risk factors associated with poor outcomes in patients with MALA.

FOOTNOTES

Author contributions: Juneja D contributed to acquisition of data, analysis and interpretation of data, drafting the article, final approval; Nasa P contributed to acquisition of data, analysis and interpretation of data, drafting the article, final approval; Jain R contributed to interpretation of data, revising the article, final approval.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: India

ORCID number: Deven Juneja 0000-0002-8841-5678; Prashant Nasa 0000-0003-1948-4060; Ravi Jain 0000-0001-9260-479X.

S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 August 15; 13(8): 665-667

DOI: 10.4239/wjd.v13.i8.665

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Loss of skeletal muscle mass is not specific to type 2 diabetes

Bo Zhou, Ying-Qi Jin, Lian-Ping He

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kobyliak N, Ukraine; Ulasoglu C, Turkey

Received: March 22, 2022
Peer-review started: March 22, 2022
First decision: May 30, 2022
Revised: May 30, 2022
Accepted: July 6, 2022
Article in press: July 6, 2022
Published online: August 15, 2022

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Abstract

Skeletal muscle is a massive insulin-sensitive tissue in the body. Loss of muscle mass is associated with mitochondrial dysfunction, and is often a result of diabetes. Insulin deficiency or insulin resistance can only be seen as reduced skeletal muscle mass. Diabetes is caused by insulin deficiency or insulin resistance; however, insulin resistance is not unique to diabetics. Insulin resistance also exists in many diseases.

Key Words: Diabetics; Insulin deficiency; Insulin resistance; Skeletal muscle mass

Core Tip: Insulin resistance is present in hypertension, and in this case, loss of skeletal muscle mass occurs. At the same time, insulin resistance also results in obesity, and in this case, there is also a reduction in skeletal muscle mass. Loss of skeletal muscle mass can occur in many diseases.

Citation: Zhou B, Jin YQ, He LP. Loss of skeletal muscle mass is not specific to type 2 diabetes. *World J Diabetes* 2022; 13(8): 665-667
URL: https://www.wjgnet.com/1948-9358/full/v13/i8/665.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i8.665

TO THE EDITOR

We read with great interest the study by Chen LY *et al*[1] which discovered that there is a relationship between loss of skeletal muscle mass and the presence of diabetic mellitus in males, but not in females. The findings have positive implications for the treatment and prevention of diabetes. Nonetheless, it appears to me that there are still some issues worth rethinking.

In the study, loss of skeletal muscle mass was shown to be associated with diabetes in men; however, the loss of skeletal muscle mass is not unique to diabetes. High insulin resistance occurs in both type 2 diabetes and high blood pressure. Insulin resistance plays a major role in the development of hypertension. Previous animal studies have also found that the spontaneously hypertensive rat manifests insulin resistance[2]. At the same time, there is a loss of skeletal muscle mass in insulin-resistant diseases. Skeletal muscle is the largest insulin-sensitive tissue in the body. Decreased muscle mass is associated with mitochondrial dysfunction and increased fat infiltration. This leads to a decrease in glucose processing capacity. Therefore, loss of skeletal muscle mass is also associated with hypertension.

In addition, insulin resistance also appears in adolescent obesity. Lipid accumulation is evident in skeletal muscles in adolescents with obesity. Intermuscular fat may impair insulin action through reducing blood flow to muscles[3,4]. Obesity is associated with biological dysfunction in skeletal muscles[5]. Sarcopenic obesity is a symptom of obesity with loss of muscle mass and physical dysfunction. Obesity can cause several biological dysfunctions, including insulin resistance, mitochondrial dysfunction, and inflammation. These changes further aggravate skeletal muscle loss and physical dysfunction. There is a study that shows that in the early stages of juvenile obesity development, the microvasculature and prefrontal cortex exhibit impaired insulin signaling[6]. This study suggests that obesity has insulin resistance. At the same time, there is a loss of skeletal muscle mass in insulin-resistant diseases. This further suggests that skeletal muscle mass loss is not unique to diabetes.

In summary, decreased skeletal muscle mass occurs in both hypertension and obesity. Insulin resistance is not just a loss of skeletal muscle mass. Loss of skeletal muscle mass is also present in many diseases and is not a specific feature of diabetes. More research is needed to determine the relationship between reduced skeletal muscle mass and diabetes.

FOOTNOTES

Author contributions: Zhou B and He LP came up with ideas and constructs; Zhou B and Jin YQ wrote the manuscript; He LP approved the main conceptual ideas and made corrections; all authors provided final edits and approved the manuscript.

Conflict-of-interest statement: Every author stated that there is no commercial, professional, or personal conflict of interest relevant to the study and hereby proves that it complies with the principles of publishing ethics.

Country/Territory of origin: China

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S-Editor: Wang LL
L-Editor: Filipodia
P-Editor: Wang LL

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Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com

ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 September 15; 13(9): 668-801

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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Monthly Volume 13 Number 9 September 15, 2022

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ABOUT COVER

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AIMS AND SCOPE

The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJD* as 4.560; IF without journal self cites: 4.450; 5-year IF: 5.370; Journal Citation Indicator: 0.62; Ranking: 62 among 146 journals in endocrinology and metabolism; and Quartile category: Q2.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

PUBLICATION DATE
September 15, 2022

World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 668-682

DOI: 10.4239/wjd.v13.i9.668

ISSN 1948-9358 (online)

REVIEW

Nonalcoholic fatty liver disease and diabetes

Maria Irene Bellini, Irene Urciuoli, Giovanni Del Gaudio, Giorgia Polti, Giovanni Iannetti, Elena Gangitano, Eleonora Lori, Carla Lubrano, Vito Cantisani, Salvatore Sorrenti, Vito D'Andrea

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Hua J, China; Luo LY, China; Yang M, United States; Zhang XL, China

Received: March 30, 2022
Peer-review started: March 30, 2022
First decision: April 25, 2022
Revised: May 3, 2022
Accepted: August 6, 2022
Article in press: August 6, 2022
Published online: September 15, 2022

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the world and represents a clinical-histopathologic entity where the steatosis component may vary in degree and may or may not have fibrotic progression. The key concept of NAFLD pathogenesis is excessive triglyceride hepatic accumulation because of an imbalance between free fatty acid influx and efflux. Strong epidemiological, biochemical, and therapeutic evidence supports the premise that the primary pathophysiological derangement in most patients with NAFLD is insulin resistance; thus the association between diabetes and NAFLD is widely recognized in the literature. Since NAFLD is the hepatic manifestation of a metabolic disease, it is also associated with a higher cardiovascular risk. Conventional B-mode ultrasound is widely adopted as a first-line imaging modality for hepatic steatosis, although magnetic resonance imaging represents the gold standard noninvasive modality for quantifying the amount of fat in these patients. Treatment of NAFLD patients depends on the disease severity, ranging from a more benign condition of nonalcoholic fatty liver to nonalcoholic steatohepatitis. Abstinence from alcohol, a Mediterranean diet, and modification of risk factors are recommended for patients suffering from NAFLD to avoid major cardiovascular events, as per all diabetic patients. In addition, weight loss induced by bariatric surgery seems to also be effective in improving liver features, together with the benefits for diabetes control or resolution, dyslipidemia, and hypertension. Finally, liver transplantation represents the ultimate treatment for severe nonalcoholic fatty liver disease and is growing rapidly as a main indication in Western countries. This review offers a comprehensive multidisciplinary approach to NAFLD, highlighting its connection with diabetes.

Key Words: Bariatric surgery; Diabetes; Hepatic steatosis; Liver fibrosis; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis

Core Tip: Nonalcoholic fatty liver disease is the most common liver disease worldwide, characterized by fat accumulation in the hepatic parenchyma, with a range of different stages from mild inflammation to severe fibrosis. There is a biunivocal relationship with type 2 diabetes, with important consequences in terms of cardiovascular risk, which seems to also have occurred during the coronavirus disease 2019 pandemic. This review focuses on the pathogenesis, clinical aspects, and treatment, providing guidance for a non-invasive diagnosis and preferred therapy, medical and/or surgical.

Citation: Bellini MI, Urciuoli I, Del Gaudio G, Polti G, Iannetti G, Gangitano E, Lori E, Lubrano C, Cantisani V, Sorrenti S, D'Andrea V. Nonalcoholic fatty liver disease and diabetes. *World J Diabetes* 2022; 13(9): 668-682
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/668.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.668

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease worldwide[1] and represents a clinico-histopathologic entity with features mimicking alcohol-induced liver injury, but occurring, by definition, in patients with little or no history of alcohol consumption. Its prevalence reaches up to 25%-30%[2,3] of the worldwide population, with approximately 2 billion of individuals being affected[4].

NAFLD includes a different variety of findings, ranging from hepatocyte fat accumulation without concomitant inflammation or fibrosis (simple hepatic steatosis), to hepatic steatosis with a necro-inflammatory component (steatohepatitis), which may or may not have associated fibrosis. Nonalcoholic steatohepatitis (NASH) may progress to cirrhosis in up to 20% of patients[5,6], and it is a leading cause of cryptogenic cirrhosis[7].

The cause of NAFLD has not been fully elucidated and is considered multifactorial. A two-hit model of NAFLD development was originally proposed. The first consists of hepatic steatosis, which then sensitizes the liver to a progressive injury and is mediated by "second hits" as inflammatory cytokines, adipokines, and oxidative stress. Together they lead to steatohepatitis and fibrosis[8]. Currently, the two-hit hypothesis has been replaced by the "multiple hit" theory, which recognizes the following components in NAFLD pathophysiology: insulin resistance, obesity, gut microbiota, and environmental and genetic factors[9].

The aim of this review is to report, from a comprehensive multidisciplinary perspective, the pathogenesis, diagnosis, and treatment of NAFLD, highlighting its relationship with diabetes.

PATHOGENESIS

The key concept of NAFLD pathogenesis is excessive triglyceride hepatic accumulation as a result of an imbalance between free fatty acid (FFA) influx and efflux[10]. This can occur from the excessive importation of FFAs from the adipose tissue; diminished hepatic export of FFA, possibly secondary to reduced synthesis or secretion of very low-density lipoprotein; or the impaired beta-oxidation of FFA. The pathogenesis and evolution of NAFLD are depicted in Figure 1.

Strong epidemiological, biochemical, and therapeutic evidence supports the premise that the primary pathophysiological derangement in most patients with NAFLD is insulin resistance. Resistance to the action of insulin results in important changes in lipid metabolism. These include enhanced peripheral lipolysis, increased triglyceride synthesis, and increased hepatic uptake of fatty acids. Each of these may contribute to the accumulation of hepatocellular triglycerides, which in turn results in a preferential shift from carbohydrate to FFA beta-oxidation, an occurrence that has been demonstrated in patients with insulin resistance[11]. The association of liver steatosis and metabolic dysfunction is so strict that a new definition was recently proposed to define this entity, namely "metabolic (dysfunction)-associated fatty liver disease" (MAFLD)[12].

The excessive inflow of triglycerides to the liver leads to inflammation, reactive oxygen species (ROS) formation, hepatocyte impaired function, and lipotoxicity. Hepatocellular cells injury activates apoptotic pathways, ultimately causing cellular death. This results in the progression from noninflammatory

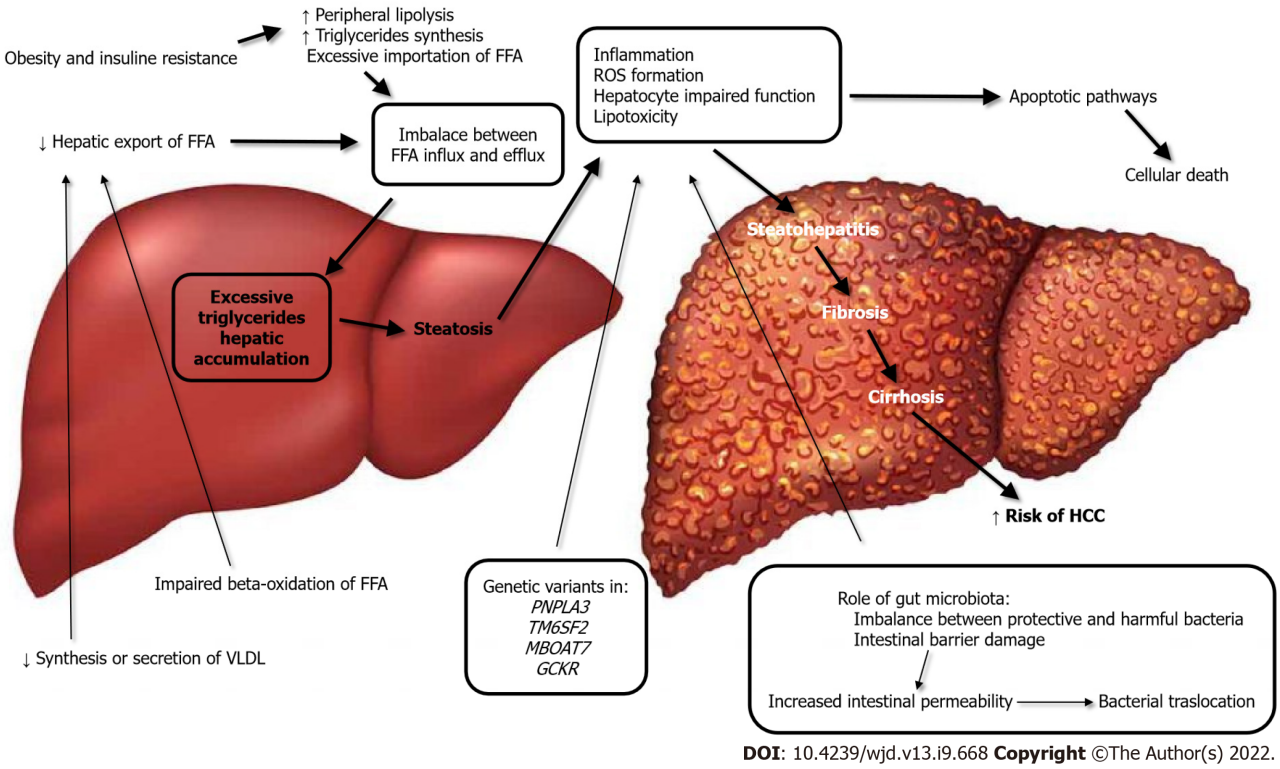

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Figure 1 Pathogenesis and evolution of nonalcoholic fatty liver disease. FFA: Fatty free acids; HCC: Hepatocellular carcinoma; ROS: Reactive oxygen species; VLDL: Very low-density lipoprotein.

isolated steatosis to the development of nonalcoholic steatohepatitis, with a risk of further evolution to fibrosis, cirrhosis and, worst-case scenario, to the development of hepatocellular carcinoma[9,13]. In this regard, the major role of mitochondrial dysfunction in the genesis of NAFLD has emerged in recent years; in fact mitochondria are responsible for the β-oxidation of FFAs and controlling the tricarboxylic acid cycle. Furthermore, mitochondria favor cell adaption to oxidative stress, mitigating the effects of ROS production[14].

Intestinal microbes have also been implicated as a potential source of hepatotoxic oxidative injury, and changes in the microbiome play a role in the lipotoxicity and pathogenesis of NAFLD[15,16].

The specific composition of gut microbiota may play a role in both the inflammatory and fibrosis responses in patients with NAFLD. The imbalance between protective and harmful bacteria, such as altered *Firmicutes/Bacteroidetes* ratio, relative abundance of alcohol-producing bacteria, growth of harmful genera, and lack of protective genera, together predispose[17] to damage of the intestinal barrier. The consequent epithelial disruption leads to an altered immune reaction and activation of inflammatory pathways, as a response to the bacterial products, namely short-chain fatty acids, trimethylamine N-oxide, and secondary bile acids[18]. Damage of the intestinal membrane finally results in impaired transport across the mucosa, increasing the filtration of bacterial lipopolysaccharides and thus further contributing to NAFLD development[17,19].

In terms of genetic risk factors, there is also a role in the development of NAFLD. Studies on twins have demonstrated a strong hereditary correlation, estimated to be approximately 50%, to both hepatic fat content and hepatic fibrosis[4]. It is recognized that at least four genetic variants in four different genes (PNPLA3, TM6SF2, MBOAT7, and GCKR) are responsible for the encoding of hepatic lipid metabolism regulatory proteins and are therefore involved in the development and progression of NAFLD[12,20].

DIABETES AND NAFLD: A WELL-ESTABLISHED RELATIONSHIP

Among type 2 diabetes (T2D) patients, the prevalence of NAFLD is more than double compared to the general population, and is estimated to be over 55%. The global prevalence of NASH in T2D patients is 37%[1]. The prevalence of NAFLD in T1D is reportedly between 10% and 20%[21,22].

The association between T2D and NAFLD is widely recognized in the literature[23-26]. T2D is itself a risk factor for the development of NAFLD, and seems to accelerate the progression of liver disease[1,27]. On the other hand, NAFLD is a risk factor for the development of T2D and its complications[22,23,27-29]. In fact, NAFLD gives a two-fold increased risk of incident diabetes over a course of about 5 years

[23,30], and the risk of patients affected by liver steatosis to develop diabetes increases in parallel to the extent of steatosis severity[30], becoming even higher when the fibrosis is advanced[23,30].

A study on 2020 participants, with a 10-year follow-up, observed that the fatty liver index (FLI), an indirect assessment used to quantify the amount of hepatic fat with a mathematical formula, predicts incident risk of developing T2D and glycemic alterations preceding diabetes. Individuals with a high FLI had an increased risk of developing diabetes, and among these high FLI patients, overweight and obese people had a risk that increased by more than 10- and 15-fold compared to similar body mass index-matched people but lower FLI[31]. Similarly, another study on 28991 pre-diabetic patients with a 3-year follow-up found that high FLI is a risk factor for developing diabetes, even in nonobese patients [32]. Of note, NAFLD predicts the development of metabolic syndrome over a period of less than 5 years[33], and metabolic syndrome is considered a risk factor for T2D.

NAFLD is associated with the development of macrovascular and microvascular complications in T2D patients, including chronic kidney disease (CKD)[29], retinopathy and autonomic neuropathy, although the results across studies are not completely concordant[34,35]. Liver fibrosis is also independently associated with macrovascular and microvascular complications in diabetic patients[36], and although T2D is a well-known risk factor to CKD, NAFLD predicts deterioration of renal function even in healthy subjects.

As per dietary advice, adherence to a Mediterranean diet is inversely associated with NAFLD and prevents the development of T2D and cardiovascular disease (CVD) in patients with NAFLD over a 10-year span[37], whereas the low adherence to these food habits is associated with diabetes and CVD onset in NAFLD patients[38]. Virtually, most studies assessing liver fat content have reported positive results after very low-calorie diets and ketogenic diets. While it is acknowledged that weight loss is associated with amelioration of NAFLD, less is known about the effect of macronutrient distribution on such outcomes. Carbohydrate restriction, with its well-established role in modulating insulin levels, and the newly proposed pathway involving the microbiome shift with increased folate production, likely plays a primary role in the reported effectiveness of ketogenic diets towards NAFLD[39].

Figure 2 summarizes the pathophysiological link between NAFLD and T2D.

DIABETES, NAFLD, AND CARDIOVASCULAR RISK

CVD is among the leading causes of death worldwide[40], and the prevention of cardiovascular events is crucial from a global health perspective.

Atherosclerotic CVD is the major cause of morbidity and mortality in diabetic patients[41]. CVD comorbidities often present in diabetic patients as hypertension and dyslipidemia, are additive risk factors for cardiovascular events. T2D is a recognized cardiovascular risk factor as well, and NAFLD contributes independently to CVD[42].

Since NAFLD is the hepatic manifestation of a metabolic disease, it is also associated with a higher cardiovascular risk[43]. A recent meta-analysis assessed the long-term higher risk of fatal and nonfatal CVD events, observing an increase across steatosis stages, reaching the maximum when fibrosis was present[44]. NAFLD is also significantly associated with hypertension[45] and heart failure[46], thus significantly increasing the overall mortality risk[46]. In a retrospective study comparing more than 900 subjects affected either by NAFLD or AFLD or with normal liver appearance on computed tomography, fatty liver independently from the cause of the steatosis was associated with a higher cardiovascular risk [47]. Since NAFLD is a dynamic entity, it is, by definition, subject to variation over time. In the same study, Lee *et al*[47] evaluated 3 million subjects for NAFLD with FLI for a minimum of four times, between 2009 and 2013, concluding that higher persistent FLI led to a higher mortality rate for all causes, myocardial infarction, and stroke. These results were confirmed after correcting for many possible confounders such as age, sex, smoking, alcohol consumption, income, dyslipidemia, body mass index, diabetes, hypertension, and physical activity[47].

As already discussed, diabetes and NAFLD are often associated; thus they may act synergistically to maximally increase cardiovascular risk[48]; the higher incidence of CVD in diabetic patients with steatosis compared to diabetic patients without steatosis[48] seems to confirm this detrimental association.

A study on > 130000 T2D patients with a hospital record of NAFLD or AFLD, and no record of any other liver disease, showed an increased risk for recurrent CVD, cancer, and mortality for all causes[49]. Patients with a history of hospital admission and fatty liver were younger than those without liver disease[50]. Of note, similar to what happens in healthy subjects and T2D patients, even in T1D patients, NAFLD increases the cardiovascular risk[51].

Figure 3 illustrates the association of T2D and NAFLD with multiple morbid conditions; thus the coexistence and interaction of the two, further exacerbates the prognosis of each.

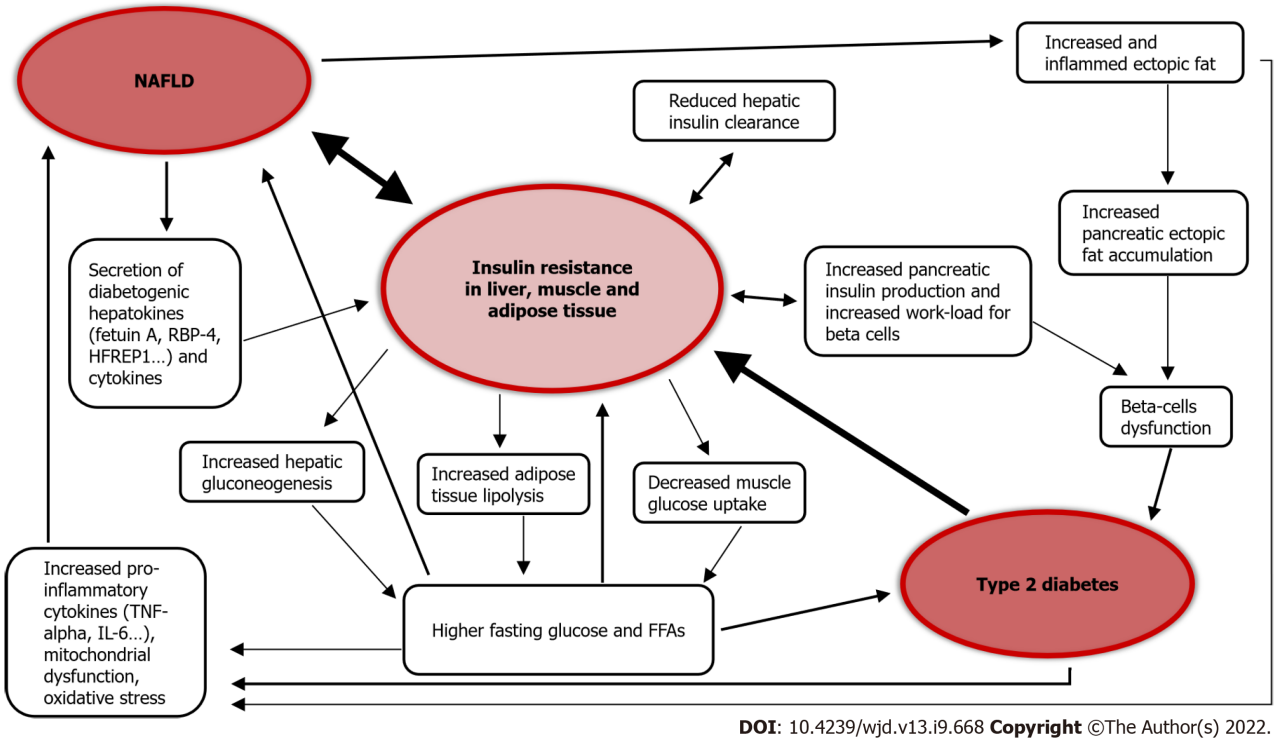

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Figure 2 The link between nonalcoholic fatty liver disease and diabetes pathogenesis. Nonalcoholic fatty liver disease increases the risk of developing type 2 diabetes mainly through worsening insulin resistance and increasing gluconeogenesis. By contrast, type 2 diabetes increases the risk of developing liver steatosis and fibrosis through insulin resistance, oxidative stress, and inflammatory cytokines.

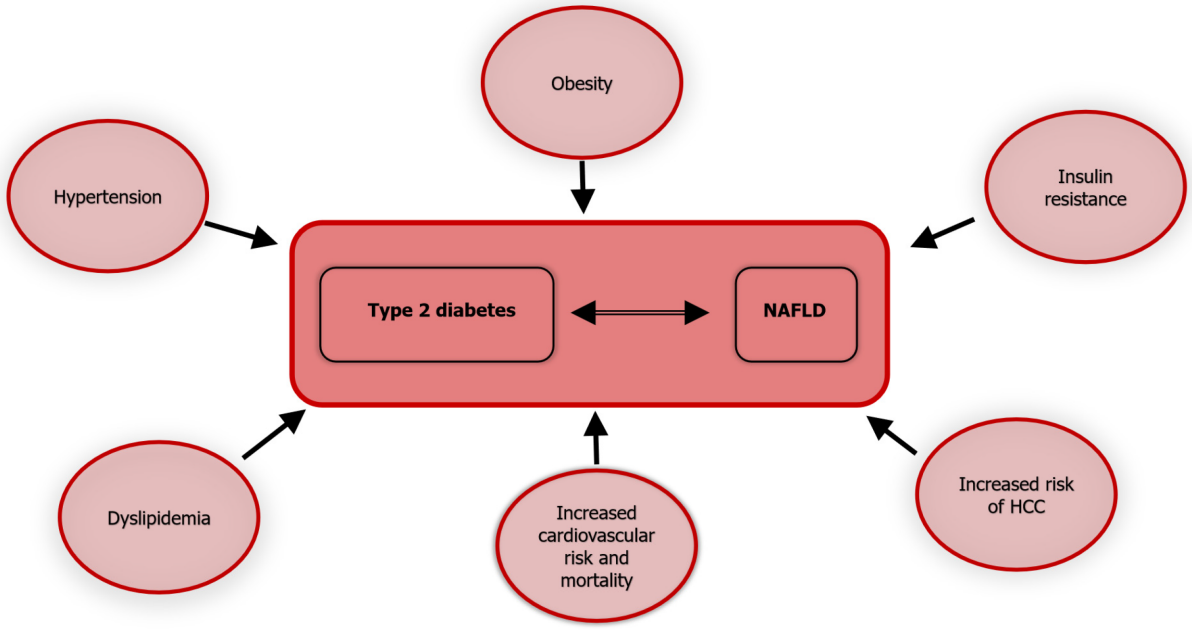

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Figure 3 Type 2 diabetes and nonalcoholic fatty liver disease are both associated with multiple metabolic and cardiovascular morbidities. Furthermore, the presence of one increases the risk to develop the other and thus exacerbating the overall prognosis. HCC: Hepatocellular carcinoma; NAFLD: Nonalcoholic fatty liver disease.

DIABETES, NAFLD, AND CORONAVIRUS DISEASE 2019

From the very beginning of the severe acute respiratory syndrome coronavirus 2 pandemic, diabetes has shown an association to this virus infection. In fact, a study on 5700 patients admitted to 12 hospitals in the New York City area demonstrated that the most common comorbidities in admitted coronavirus

disease 2019 (COVID-19) patients were hypertension (56.6%), obesity (41.7%), and diabetes (33.8%)[52]. Diabetes prevalence in COVID-19 patients is high, varying from 15%, in a pool of more than 23000 patients[53], up to almost 40% in another study on 200 hospitalized patients[54].

Diabetic patients have a higher risk of contracting COVID-19[55], a higher risk of hospitalization[54] and mortality[56].

NAFLD is also associated to COVID-19[57], to its severity progression, risk of intubation, dialysis and use of vasopressors[58], although in contrast, some authors[59-61] did not observe a higher risk of severe COVID-19 and intensive care unit access for NAFLD patients.

A longer viral shedding time[62] and a higher mortality for COVID-19 in NASH patients with advanced fibrosis[63] have also been reported.

NAFLD DIAGNOSIS

NAFLD diagnosis is based on three criteria: (1) Absence of significant alcohol intake; (2) presence of hepatic steatosis; and (3) exclusion of other causes of liver disease.

Some clinical biomarkers are used to screen for or diagnose NAFLD, used in complex algorithms for risk stratification. They aim to combine various conditions, such as arterial hypertension with laboratory exams, like transaminases, to predict outcomes of the liver disease, but as single markers, they only provide poor sensitivity and specificity. Yet, their overall performance is limited, with further studies needed to transfer the initial thought cut-off values into the real clinical scenario[64].

It can therefore be asserted that due to the lack of available noninvasive methods to confirm the diagnosis of NAFLD, liver biopsy remains the gold standard to classify steatosis, and NASH. However, biopsy has limitations[65]; namely it is invasive, subject to sampling variability and observer-dependence, and most importantly, carries risks. Therefore, it is not offered to routinely assess the amount of fatty liver in NAFLD patients who may have simple steatosis, as reported in the majority of cases[6].

As previously mentioned, since NAFLD is a dynamic entity[47], varying through lifetime, imaging methods remain the most widely utilized tools to assess NAFLD patients and quantify the relative hepatic steatosis.

NAFLD IMAGING

To date, various imaging methods have been utilized: ultrasonography, CT, magnetic resonance imaging (MRI), and magnetic resonance spectroscopy (MRS). More recently, other diagnostic tools measuring liver stiffness have entered clinical practice, in view of their practical utility, as reported in Table 1.

Ultrasound

Conventional B-mode ultrasound (US) is the most widely used imaging modality for the noninvasive evaluation of hepatic steatosis, as first-line diagnostic imaging procedure, according to clinical practice guidelines[66]. Fatty liver infiltration is characterized by hyperechogenicity of the parenchyma and increasing attenuation of US waves in deeper parts, specifically where there is increasing steatosis[67]. However, US evaluation of fatty livers is based on the operator's experience; in comparison to histology as reference standard, the overall sensitivity and specificity of B-mode US are, respectively, 84.8% and 93.6%, with 0.93 accuracy[68].

US elastography quantitatively evaluates liver stiffness. Two broad categories of imaging-based sonoelastography are currently in clinical use: strain elastography, which is influenced by the operator or physiologic forces that produce tissue deformation; and shear wave elastography (SWE), which instead results from the acoustic radiation force of the tissue displacement[69,70].

Fibroscan uses transient US elastography (TE) to measure hepatic elasticity by quantifying the shear wave velocity with ultrasonic echo pulses from low-frequency vibrations that are transmitted into the liver[71,72]. Since patients with > 66% steatosis at liver biopsy have a false-positive higher rate, *via* the Fibroscan XL probe it is also possible to investigate obese patients, given that during TE the transmission of a mechanical wave through the skin and subcutis could cause technical failure and unreliable measurements[73].

Controlled attenuation parameter (CAP) is another technique implemented on the Fibroscan device. The principle of CAP is to measure the acoustic attenuation in liver of shear waves generated by the probe. The amount of fat deposited in the liver can be inferred from the degree of attenuation[74]. In a multimodality study in patients with biopsy-proven NAFLD, it was shown that using a threshold of 261 dB/m CAP the methodic accuracy was 0.85 (95% confidence interval of 0.75–0.96) for steatosis diagnosis [75].

Table 1 Pros and cons of imaging modalities to assess hepatic steatosis

Modality	Pros	Cons
US B-Mode	Lack of ionizing radiation	No panoramic view
	Less expensive	Operator dependency
	Repeatable	Limited accuracy diagnosing mild hepatic steatosis
	Fast	Rather qualitative nature
	Can be performed at the bedside (no need to transport the patient)	Non simple steatosis/NASH differentiation
	Useful also for identification of other pathology such as liver lesions	
QUS	Same as US B-Mode	Not always available
	Quantitative and semiquantitative fat evaluation (less operator sensitive)	Need to buy newer machines and software
Fibroscan	Quantitative evaluation (less operator sensitive)	Expensive equipment that doesn't supply imaging evaluation
	Lack of ionizing radiation	
	Fast	
	Can be performed at the bedside (no need to transport the patient)	
CT	Fast	Ionizing radiation
	Panoramic view	Limited accuracy diagnosing mild hepatic steatosis
	Volumetric rendering	Non simple steatosis/NASH differentiation
	High spatial resolution	
	Quantitative density evaluation	
MRI	Highly accurate and reproducible for measuring hepatic fat	Expensive
	Panoramic view	Examination time
	Lack of ionizing radiation	Software not always available
	Quantitative fat evaluation	
MRS	Highly accurate and reproducible for measuring hepatic fat	Expensive
	Panoramic view	Examination time
	Lack of ionizing radiation	Software not always available
	Quantitative fat evaluation	Evaluation of small portion of the liver
		Expertise required for data acquisition and analysis

CT: Computed tomography; MRI: Magnetic resonance imaging; MRS: Magnetic resonance spectroscopy; NASH: Nonalcoholic steatohepatitis; QUS: Quantitative ultrasound; US: Ultrasound.

Two-dimensional SWE is an US technique providing visualization of viscoelastic properties of soft tissues in real time[76]. These techniques employ acoustic radiation force impulses that induce tissue motion at a microscopic level, which in turn produces tissue shear waves. The shear waves are related to tissue stiffness under simple assumptions, expressed as Young's module[77].

In the last several years, quantitative US measures, such as the ultrasonic attenuation coefficient and backscatter coefficient, derived from the raw radiofrequency echo data, have been considered a noninvasive tool for the objective assessment of hepatic steatosis[78].

A general limitation of all US-based methods evaluating liver fat content, including CAP, is that sonography exploits the attenuation of the propagated and reflected waves. While liver fat attenuates sound waves, many other liver pathologies such as hepatitis, hemochromatosis or fibrosis can also affect sound waves in the same manner[79].

CT

CT evaluation of hepatic steatosis is based on the attenuation values of the liver parenchyma, assessed as Hounsfield units (HU), in association with tissue composition. The attenuation value of fat (approximately -100 HU) is much lower than that of soft tissue, so hepatic steatosis lowers the attenuation of liver parenchyma. Some studies have reported that contrast-enhanced venous CT and nonenhanced CT

have comparable diagnostic accuracy for hepatic steatosis[80]; however, nonenhanced CT is usually preferred to avoid the potential errors of contrast-enhanced CT caused by variations in hepatic attenuation related to contrast injection methods and scan times. The two CT indexes most frequently used to assess steatosis are the absolute liver attenuation value (*i.e.* HU-liver) and the attenuation difference between the liver and spleen.

CT is accurate for the diagnosis of moderate-to-severe steatosis but is not as accurate for detecting mild steatosis. The threshold values of CT indices for the diagnosis of hepatic steatosis are quite variable, depending on the methods and populations used[81-83]. Furthermore, some factors may affect hepatic attenuation on CT, such as the presence of excess iron in the liver and ingestion of certain drugs such as amiodarone[84].

Magnetic resonance

While CT and US assess hepatic steatosis through proxy parameters (echogenicity and attenuation, respectively), MRI can more directly measure the amount of hepatic fat, in fact it is an imaging modality with a rich range of contrast mechanisms detecting and quantifying hepatic fat content through the measurement of proton signals present in water and fat[85].

There are conventional MRI methods providing qualitative estimates of hepatic steatosis and fully quantitative MRS and MRI methods that allow for an accurate and precise measurement of hepatic fat content[86-88].

MRS and chemical shift-encoded MRI, when performed in expert hands, can serve as confounder-corrected methods able to discern the number of fat-bound protons divided by the amount of all protons in the liver, including fat- and water-bound protons[89].

To date, MRI especially with the techniques reported above, represent the noninvasive gold standard evaluation of these patients; however, US is broadly gaining popularity.

PREVENTION AND TREATMENT

NAFLD treatment depends on the severity of the disease, ranging from a more benign condition of nonalcoholic fatty liver to nonalcoholic steatohepatitis, which is at the more severe end of the spectrum. However, there are some measures that can be applied to all patients. These include the following. (1) Abstinence from alcohol: evidence shows that in NAFLD patients, there is no liver-safe limit of alcohol intake[90]. Heavy alcohol use is well-known to be associated with hepatic steatosis, hepatic injury, and progression of parenchymal fibrosis[91], but even low alcohol consumption in individuals with metabolic abnormalities could be harmful, thus abstinence from alcohol for patients with NAFLD is always recommended. (2) Immunizations: for patients without serologic evidence of immunity, vaccination for hepatitis A virus and hepatitis B virus is recommended, and, in general, standard, age-appropriate immunizations for all patients[7]. (3) Modification of risk factors for CVD: For patients with hyperlipidemia, lipid-lowering therapy; for patients with diabetes, optimizing blood glucose control[9].

For patients with NASH and T2D, the presence of the liver disease can inform the choice of glucose lowering therapy, and although this is typically with metformin, the beneficial impact on liver histology with certain other insulin-sensitizing agents could be of note when choosing a second-line agent in NASH patients, if metformin is contraindicated or in need of additional glucose-lowering therapy[33, 35]. In this setting, pioglitazone and GLP-1 receptor agonists (*e.g.*, liraglutide, semaglutide) are reasonable options[92] and the apparent benefit of certain insulin-sensitizing agents for NAFLD is likely related to the role that insulin resistance plays in the development of NAFLD[9].

For patients with biopsy-proven NASH and fibrosis stage 2 but without diabetes, the use of vitamin E (800 international units per day) is suggested. The antioxidant, anti-inflammatory, and anti-apoptotic properties of vitamin E accompanied by the ease-of-use and exceptional tolerability have made vitamin E a pragmatic therapeutic choice in nondiabetic patients with histologic evidence of NASH[93].

In every case, weight loss is the primary therapy for most patients with NAFLD. It can lead to improvement in liver biochemical tests, liver histology, serum insulin levels, and quality of life[94-96].

Several studies have suggested that weight loss of at least 5% of body weight is necessary to improve hepatic steatosis, although the long-term benefits of such weight loss are unknown. In a meta-analysis of eight trials including 373 patients, losing 5% of body weight resulted in improvement in hepatic steatosis, while losing of 7% of body weight was associated with improvement in NALFD activity score, which is used to grade disease activity[97].

Unfortunately, only less than 10% of patients that try to lose weight with lifestyle modifications, including diet and physical activity, achieve this target at 1-year, and fewer maintain the weight loss at 5 years[98]. Bariatric surgery is an option that may be considered in those who fail to lose weight by lifestyle changes.

Although weight loss seems to be the main mechanism, bariatric surgery has been shown to improve also liver histology and fibrosis secondary to NASH, in addition to other benefits including an improvement or resolution of T2D mellitus, dyslipidemia, and hypertension, and a reduction of cardiovascular morbidity or mortality[99-101].

A meta-analysis of 10 studies showed that the bariatric surgery group had significantly lower odds of major adverse cardiovascular events as compared to no surgery (odds ratio = 0.49; 95% confidence interval: 0.40-0.60; $P < 0.00001$; $I^2 = 93\%$) suggesting the benefit of bariatric surgery in reducing the occurrence of serious events in patients with obesity and CVDs[102].

In the SPLENDOR study of 1158 patients with histologically confirmed NASH and obesity, bariatric surgery (gastric bypass or sleeve gastrectomy) was associated with a much lower 10-year cumulative incidence of major adverse liver outcomes (2.3% *vs* 9.6%) and major cardiovascular events (8.5% *vs* 15.7%) compared with nonsurgical management[103].

Weight reduction due to bariatric surgery causes inflammatory changes in patients with obesity. After gastric bypass there is a proven reduction of hepatic expression of factors involved in the progression of liver inflammation (macrophage chemoattractant protein 1, and interleukin-8) and fibrogenesis [transforming growth factor-β1, tissue inhibitor of metalloproteinase 1, α-smooth muscle actin, and collagen-α1(I)][104], a significant decrease in mean NAFLD fibrosis score after Roux-en-Y gastric bypass (RYGB) and resolution rate of 55% of severe fibrosis in 12-mo observation[105], and, moreover, RYGB contributes to significant reduction in NAFLD activity score, steatosis, inflammation and liver ballooning during 1-year observation[106].

In a long-term follow-up of patients with NASH who underwent bariatric surgery, Lassailly *et al*[107] observed resolution of NASH in liver biopsies from 84% of patients 5 years later. The reduction of fibrosis is progressive, beginning during the 1st year and continuing through 5 years[107].

Among recently available surgical methods, RYGB and laparoscopic sleeve gastrectomy (LSG) are the most performed worldwide. The remaining question is whether RYGB or LSG is more effective[108].

A systematic review and meta-analysis performed by Baldwin *et al*[109] compared RYGB and LSG using separate criteria: transaminases concentration, NAFLD activity score and NAFLD fibrosis score. Overall, both RYGB and LSG significantly improved liver enzymes, NAFLD activity score, and NAFLD fibrosis score postoperatively. Direct comparisons of RYGB to LSG in any of the criteria failed to demonstrate superiority[109]. These findings, without any significant difference between the two groups, are confirmed in other studies[110,111].

Even if the role of bariatric surgery in the treatment of NAFLD is significant, there are some patients that will develop new or worsened features of NAFLD after a bariatric procedure[112]. A 5-year prospective study performed by Mathurin *et al*[113] showed that 19.8% of patients experienced fibrosis progression at 5 years follow up for unknown reason.

Aggravation of NAFLD after surgery should be kept in mind when qualifying patients for a bariatric procedure. At the extreme consequences, and when the progression of liver fibrosis is irreversible, also liver transplantation becomes an option, and indeed NASH is nowadays representing the fastest growing indication in Western countries to this kind of surgery. Yet, lifestyle modifications, as well as pharmacological strategies and tailored immunosuppression *via* a strategic multidisciplinary approach are still key to control diabetes and CVD risk in this setting, too[114].

CONCLUSION

NAFLD is intimately related to T2D and both diseases are highly prevalent worldwide, representing a public health alarm. The diagnosis and management of NAFLD in T2D is challenging, given the inherent cardiovascular risk and the underlying liver parenchymal degeneration. As well as to insulin resistance, NAFLD may be related to other hormonal alterations, quite common in patients with obesity, and potentially contributing to the onset and the worsening of steatohepatitis. A complete hormonal workout, in patients with severe NAFLD, and conversely investigation of NAFLD in patients with T2D, severe obesity or other metabolic disorders is recommended to prevent and monitor NAFLD risk.

Current medical treatments aim to mitigate insulin resistance, optimizing metabolic control and halting hepatic disease progression; yet they are still under debate for their efficacy, and new classes of drugs targeting different pathways need experimentation in the forms of randomized controlled trials, to pursue a tailor-made approach, for example assessing gut permeability and modification of individual human microbiota.

Identification of simple, inexpensive biomarkers would be also of help as an additional diagnostic tool, or to predict disease progression and response to treatment.

Surgery is considered a more advanced therapeutic option, either to improve obesity and control of the associated metabolic conditions, *via* bariatric interventions, either by substituting the cirrhotic liver *via* organ transplantation.

Future research should focus on the treatment of NAFLD, as a risk factor for developing T2D and in how to prevent and detect NAFLD progression in patients with T2D, obesity or other severe metabolic conditions.

FOOTNOTES

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 683-695

DOI: 10.4239/wjd.v13.i9.683

ISSN 1948-9358 (online)

REVIEW

A review of potential mechanisms and uses of SGLT2 inhibitors in ischemia-reperfusion phenomena

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Specialty type: Cardiac and cardiovascular systems

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Athyros VG, Greece; Ved J, India

Received: April 25, 2022
Peer-review started: April 25, 2022
First decision: May 30, 2022
Revised: June 13, 2022
Accepted: August 16, 2022
Article in press: August 16, 2022
Published online: September 15, 2022

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Abstract

Recently added to the therapeutic arsenal against chronic heart failure as a first intention drug, the antidiabetic drug-class sodium-glucose cotransporter-2 inhibitors (SGLT2i) showed efficacy in decreasing overall mortality, hospitalization, and sudden death in patients of this very population, in whom chronic or acute ischemia count among the first cause. Remarkably, this benefit was observed independently from diabetic status, and benefited both preserved and altered ventricular ejection fraction. This feature, observed in several large randomized controlled trials, suggests additional effects from SGLT2i beyond isolated glycemia control. Indeed, both in-vitro and animal models suggest that inhibiting the Na^+/H^+ exchanger (NHE) may be key to preventing ischemia/reperfusion injuries, and by extension may hold a similar role in ischemic damage control and ischemic preconditioning. Yet, several other mechanisms may be explored which may help better target those who may benefit most from SGLT2i molecules. Because of a large therapeutic margin with few adverse events, ease of prescription and potential pharmacological efficacity, SGLT2i could be candidate for wider indications. In this review, we aim to summarize all evidence which link SGLT2i and ischemia/reperfusion injuries modulation, by first listing known mechanisms, including metabolic switch, prevention of lethal arrythmias and others, which portend the latter, and second, hypothesize how the former may interact with these mechanisms.

Key Words: SGLT2 inhibitors; Ischemia-reperfusion injuries; Sodium-proton exchanger; Myocardial ischemia; Immunomodulation

Core Tip: The antidiabetic drug-class sodium-glucose cotransporter-2 inhibitors (SGLT2i) showed efficacy in decreasing mortality in patients with chronic heart failure, in whom ischemia counts among the first cause. Remarkably, this benefit was observed independently from diabetic status. This feature, yielded from several randomized controlled trials, suggests additional effects from SGLT2i beyond isolated glycemia control. Indeed, previous in-vitro and animal models analyzed altogether suggests the role of the inhibition of the Na^+/H^+ exchanger, which holds a pivotal role in ischemia/reperfusion injuries. In this review, we aim to summarize evidence which associate SGLT2i and ischemia/reperfusion injuries, by first listing known mechanisms which portend the latter, and second, hypothesize how the former may interact with these mechanisms.

Citation: Quentin V, Singh M, Nguyen LS. A review of potential mechanisms and uses of SGLT2 inhibitors in ischemia-reperfusion phenomena. *World J Diabetes* 2022; 13(9): 683-695
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/683.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.683

INTRODUCTION

Although sodium-glucose cotransporter-2 inhibitors (SGLT2i) represent a decade-old drug class, the range of their indications has expanded since the first Food and Drug Administration label in 2013 in patients with type 2 diabetes[1,2]. Indeed, SGLT2i which include empagliflozin, dapagliflozin and canagliflozin are now indicated in patients with heart failure, independently from their status towards diabetes[3].

To understand how SGLT2i went from an antidiabetic to a cardioprotective treatment, one must recall how in patients with type 2 diabetes treated by SGLT2i, there were numerous observations of a decrease in heart failure events, all-cause mortality, cardiovascular mortality[4]. Furthermore, subgroup analyses confirmed that this risk decrease was consistent across a wide range of cardiovascular risk[5,6].

Hence, specific randomized controlled trials were launched to assess the hypothesis of a benefit to be treated by SGLT2i for patients with heart failure, regardless of the presence or absence of diabetes. Preliminary reports were then confirmed, and SGLT2i improved clinical outcomes in patients presenting with heart failure, be they with preserved and reduced ejection fraction[2,7-9].

Nevertheless, while the main pharmacological effect of SGLT2i is to decrease renal glucose reabsorption, thereby increasing urinary glucose excretion, the benefits observed even in non-diabetic patients question off-target mechanisms. As an illustration, in the EMPA-REG OUTCOME trial which compared empagliflozin to placebo in patients with type 2 diabetes at high risk for cardiovascular events, the proportion of acute myocardial or cerebral ischemic event was similar in both groups, however, patients in the treatment group were more likely to surviving a cardiovascular event. This element may be supportive of a cellular protective association in ischemic injury[10]. In the dapagliflozin and prevention of adverse-outcomes in heart failure trial (DAPA-HF), administration of dapagliflozin reduced risk of serious ventricular arrythmia, cardiac arrest or sudden death[11].

In the following review, we aimed to suggest several mechanisms which may explain how SGLT2i act as immunomodulators, and how they may act beyond the sole increase in urinary loss of glucose. We first described the ischemia-reperfusion injury phenomenon and then expanded on the interactions between SGLT2i and ischemia-reperfusion mechanisms. Our main assumption lied on a protective role against ischemia-reperfusion lesions, which involve an increase in functional ketones, associated with a metabolic change, an impact on sodium/hydrogen exchanger, endothelial dysfunction, inflammation biomarkers, and platelet function.

ISCHEMIA-REPERFUSION INJURY, AN OVERVIEW

While mortality of acute myocardial infarction, has been decreasing over time[12], subsequent morbidity manifested by heart failure has grown. Mitigating infarct size is a therapeutic goal which may be attained by decreasing the delay between first signs of ischemia and revascularization[13], and by managing secondary lesions.

Myocardial ischemia is often caused by the occlusion of epicardial artery resulting in the ischemia of the coronary vascular territory which it depends upon. If prolonged, it may lead to myocardial infarction, an irreversible condition[14,15]. Therefore, quickly restoring blood flow in the occluded artery is the only way to limit the extent of infarction and subsequent complications including mortality. The reperfusion phenomenon however has been associated with secondary lesions[16], responsible for additional cardiomyocyte injuries[17,18]. These additional lesions may be partly responsible of final infarction size and therefore associated with adverse outcomes as there is a link between infraction size

and long-term mortality or heart failure[19].

In cardiac surgery, these lesions are detected in 25% to 45% of patients[20]. They may be assessed by CK-MB and/or troponin levels, associated with postoperative adverse events[21]: arrythmias, myocardial stunning, low cardiac output syndrome and perioperative infarction[22]. Although, situations leading to these myocardial injuries are either unpredictable (*i.e.*, acute myocardial infarction) or unavoidable (*i.e.*, cardiac surgery), cardioprotective strategies aiming at reducing ischemia/reperfusion injury are critical[23].

Myocardial ischemia

Defined by a mismatch between supply and need in oxygen and nutrients, its consequences depend on its severity, duration and the existence of collateral circulation[24]. In normal blood flow situation, oxygen is used by mitochondrial respiratory chain to produce ATP by using fatty acids (65%), glucose (15%), lactate (15%) and amino-acids and ketones (5%). Ninety percent of produced ATP are used by cardiomyocytes for contraction and the rest for homeostasis[25]. Following arterial occlusion and oxygen supply arrest, oxidative phosphorylation by mitochondrial respiratory chain stops and metabolism becomes anaerobic with the use of anaerobic glycogenolysis, leading to formation of H^+ and lactates[26]. Hence, during ischemia, ATP is mainly produced from glucose instead of fatty acids, due to a higher energy-consumption rate of fatty acids catabolism[27]. This metabolic shift leads to the accumulation of AcylCoA and AcylCarnitine, both considered toxic for cardiomyocytes (enzymatic inhibition, alters cell membrane etc.). The small amount of produced ATP is used to maintain cellular homeostasis by using ATP-dependent ion pumps, until all ATP are depleted. Owing to ATP deficiency some cellular functions are not further ensured such as myocardial contraction, protein synthesis[28].

Then, an intracellular sodium accumulation creates a cellular oedema due to the activation of Na^+/H^+ exchanger (NHE) and inhibition of NA/K ATPase, which in turn, leads to a cytosolic calcium overload by activation of Na^+/Ca^{2+} exchanger[29,30], inhibition of SERCA[30], and increased calcium entry *via* other channels[30].

The subsequent activation of protease, lipase, nuclease[27], and mitochondrial ultrastructural damage, are associated with myocardial stunning. In normal conditions, mitochondria's membrane is impermeable to ions and proteins[22], with a channel on the inner membrane called the mitochondrial permeability transition pore (mPTP)[25]. During ischemia, this permeability transitions, opening mPTP [22], leading to mitochondrial oedema and death and release of its contents: Cytochrome c, apoptosis-inducing factor AIF, reactive oxygen species (ROS)[31,32].

ROS are highly reactive elements responsible for cellular injury because of reactions with lipids, proteins, and nucleic acids. The accumulation of xanthine and hypoxantine during ischemia[33], allows for their use by xanthine oxidase, activated during reperfusion and leading to the formation of ROS[34]. One of the many sources of hypoxanthine during ischemia, is ATP degradation by adenine nucleotide translocase which synthesize ADP, then degraded into hypoxanthine. This phenomenon increases energetic deficiency.

Reperfusion injury

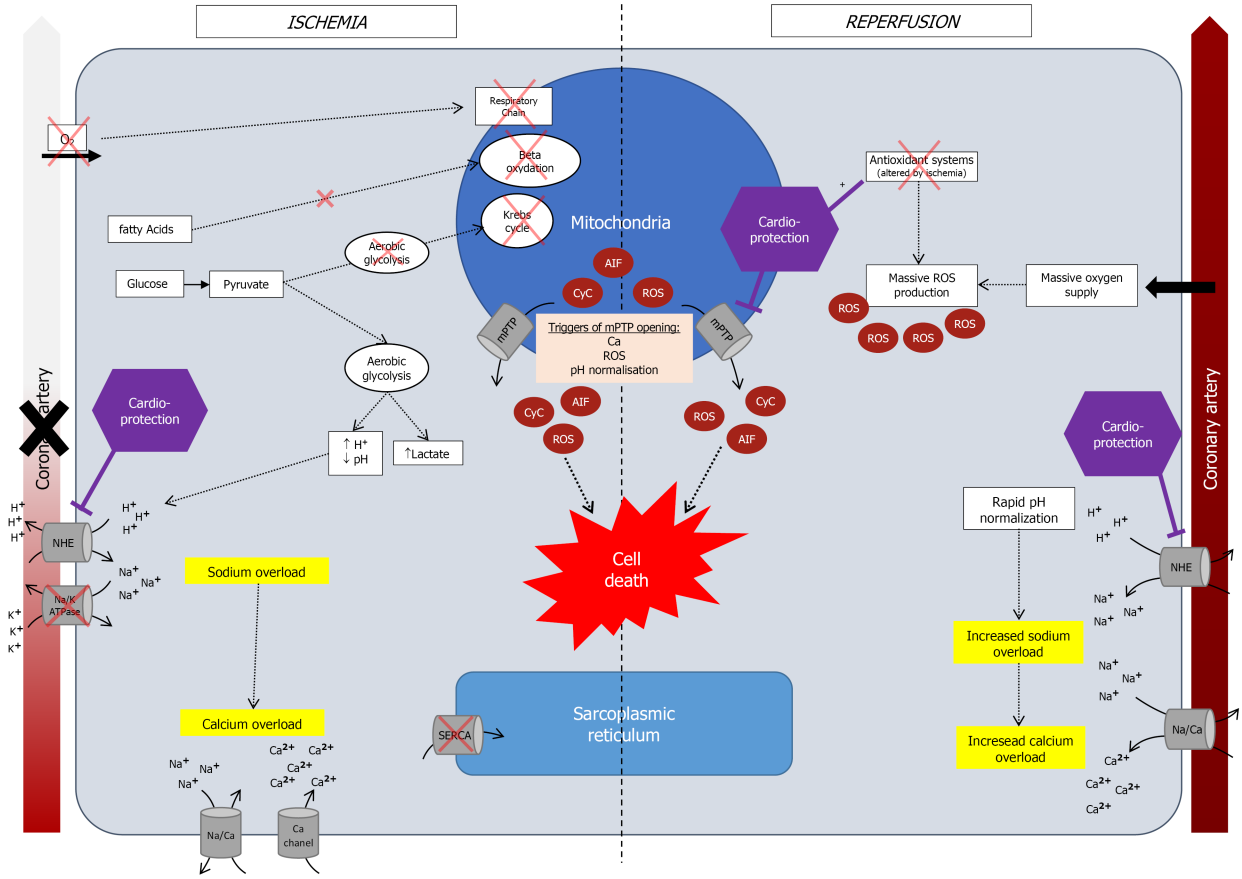
After myocardial ischemia, restoring blood flow is an emergency, and clinical guidelines all advocate for the shortest delay possible[13,25]. However, reperfusion is also associated with secondary injuries[35], due to the sudden oxygen supply which allows for the formation of superoxide anions. The mechanisms which are hypothesized include: (1) The activation of oxidative phosphorylation; (2) the activation of xanthine oxidase; and (3) local neutrophil accumulation and NADPH oxidase activation, also leading to ROS accumulation[25]. In normal conditions, superoxide anions are antagonized by antioxidant elements (catalase, superoxide dismutase, glutathione peroxidase, vitamins, *etc*.). However, in case of massive ROS production and altered defense mechanisms by ischemia, the balance is tipped off towards ROS accumulation. A graphic summary of these mechanisms is available in Figure 1.

Another mechanism of reperfusion injury is the pH paradox[25,36]**.** Reperfusion restores pH by quickly extracting accumulated H^+, by activating of NHE; yet, pH restoration has been associated with deleterious outcomes[37]. Indeed, an abrupt accumulation of Na^+ may lead to cellular oedema and calcium overload (due to a Na^+/Ca^{2+} exchanger), and since cytoplasmic acidosis inhibits the mPTP opening, rapid normalization of intracellular pH leads to mitochondrial permeability transition with mPTP reopening[27]. Hence, phenomena similar to that of ischemia may occur even though reperfusion was achieved[29].

Cardioprotective strategies

Cardioprotective strategies aim to reduce cardiomyocytes injuries, secondary to ischemia-reperfusion phenomena, and include 4 methods: preconditioning, postconditioning, remote conditioning and pharmacological treatment.

Preconditioning consists in applying cycles of brief coronary occlusion immediately before sustained occlusion. Clinical benefit has been observed in dog models, where repetitive short coronary occlusions preceding sustained occlusion resulted with an infarction smaller more delayed than that of a sustained occlusion without preconditioning[38]. While the benefit was initially observed shortly after ischemia,



DOI: 10.4239/wjd.v13.i9.683

Figure 1 Simplified physiopathology of ischemia/reperfusion injuries and cardio-protection. During ischemia, lack of oxygen leads to mitochondrial respiratory chain failure. ATP is produced mainly by using anaerobic glycolysis leading to acidosis and increase in lactate. Intracellular accumulation of H^+ activates the Na/H exchanger causing a sodium overload and calcium overload. All of these phenomenon result in mitochondrial permeability transition pore (mPTP) opening and release of reactive oxygen species (ROS), cytochrome C and AIF leading to cell death. During reperfusion, sudden oxygen supply led to massive ROS formation that are not eliminated by antioxidant systems (which have been damaged by ischemia). The rapid pH normalization increased the sodium and calcium overload (= pH paradox). mPTP opening is also increased. Cardio-protection strategies lead to inhibition of NHE, mPTP opening, or restauration of antioxidant systems. AIF: Apoptosis inducing factor; CyC: Cytochrome C; mPTP: Mitochondrial permeability transition pore; NHE: Na/H exchanger; ROS: Reactive oxygen species.

more lasting effects have been recently highlighted suggesting the role of protein synthesis [inducible nitric oxide (NO) synthase, cyclooxygenase, aldose reductase, superoxide dismutase][18]. Elements which are thought to mediate preconditioning benefit include but are not limited to adenosine, bradykinin or mechanical stretch activating various intracellular signaling pathways including RISK-pathway (increasing AKT and ERK1/2) and SAFE-pathway (increasing JAK and STAT) whose end targets are inhibition of mPTP opening, inhibition of Na/H exchanger or upregulation of antioxidant systems (superoxide dismutase, aldose reductase, *etc.*)[18,38].

Although promising, preconditioning is not reliable in clinical practice since it could not be used before acute coronary syndrome because of the brief effects of such procedure or the unpredictability of ACS. Hence, preconditioning could only be used in patient before CABG, by cross-clamping the aorta and then releasing for several minutes. Studies showed that it decreased post-operative ventricular arrhythmias, inotrope use and limited ICU stay[39].

On the other end, ischemic postconditioning consists in the same procedure, performed after the ischemic event, during reperfusion procedures. Similarly, it was associated with smaller infarct size[40, 41], a more progressive pH restoration, decreasing ROS production and calcium-induced mPTP opening, resulting in anti-apoptotic, anti-autophagic et anti-arrhythmic benefit[25].

While pre- and postconditioning aim at stimulating local anti-inflammatory pathways, remote conditioning consists in applying cycles of brief occlusion in other territories than that which is affected by ischemia (*i.e.*, neighboring coronary artery, limb). Theoretical advantages of this method lie in the fact that it may be applied at any time, is non-invasive and easily feasible. On top of the abovementioned mechanisms, additional systemic signal pathways may be involved with neuronal (peripheral sensory nerves, spinal cord, brainstems and vagal nerves) and humoral inducing a renal production of adenosine[42]. While this approach also aims at diminishing infarct size, mortality, and hospitalization for heart failure, phase III clinical trials failed to yield significant benefit, excepts in the most severe patients (cardiogenic shock or cardiac arrest)[18].

Yet, while multiple drugs have been tested, none showed clinical significance in human patients. Na/H exchangers inhibitors showed improvement in cardiovascular outcomes but increased stroke incidence[25,43,44]. Cyclosporine A, a nonspecific inhibitor of mPTP[45], promising initial results infarct, which were not translated in clinical studies[18]. Adenosine, acting as a vasodilator, was associated with pre- and postconditioning-like effects[46], through inhibition of mPTP opening[47]. Similarly, results were not conclusive in clinical trials[48]. Finally, NO was associated with potentially benefit in ischemia-reperfusion injuries by acting on oxygen consumption[49], platelet aggregation[50], leucocyte adhesion[51], and free radical scavenging[52].

These discrepancies between theoretical promises and disappointing clinical results require further research in the field, investigating novel pathways.

THE SGLT2 PATHWAY

Metabolic shift to a sparing substrate

In normal oxygenation conditions, myocardial mitochondrial oxidative metabolism exploits fatty acids (60%), glucose (30%), lactate and to a lesser degree ketones and amino acids, with a capacity to rapidly change substrates depending on workload or conditions. Under hypoxic conditions, myocardial substrate oxidation switches from free fatty acids to glucose and carbohydrate oxidation, because transformation of glucose to lactate is independent of oxygen supply[53]. During prolonged anaerobia, ketone becomes predominant as a resource. For instance, in animal models increasing the uptakes of 3-hydroxybutyrate (3HB) is associated with an improvement in cardiac function, pathologic cardiac remodeling, and oxygen consumption, whereas the capacity to oxidate substrate such as fatty acid is reduced[54]. Of note, 3HB is generated in the liver and may be used as a substrate for generating acetyl-CoA leading to increased production of NADH to drive energy transfer and ATP production.

Remarkably, in patient treated by SGLT2i, an uprising of ketone circulation was observed[55,56].

One of the hypotheses is that SGLT2i improves myocardial fuel metabolism, contractility, and cardiac efficiency by shifting catabolism away from lipids and glucose to that of ketone bodies[57]. Improved oxygen consumption and work efficiency at a mitochondrial level have been hypothesized[58]. Similarly as fasting, with the expected glucose depletion under SGLT2i, insulin-glucagon ratio is modulated, delivery of free fatty acids is increased to the liver which then stimulates ketogenesis[59]. Metabolomic profiles of patients with type 2 diabetes further support this hypothesis[55]. In addition to an expected reduction in glucose, SGLT2i increased 3HB levels suggesting an accrued utilization of ketone bodies. Moreover, increased intermediate metabolites of the urea cycle may indicate its use as well as amino-acids[55]. Remarkably, the same metabolic changes were observed in non-diabetic patients: Ferrannini *et al*[54] showed that SGLT2i reduced end-tissular glucose catabolism, accelerated lipolysis and fat oxidation. While these changes were more prominent after long-term exposition, an effect was observed as early as the first administration[53]. When compared to serum profiles of patients under corticoids treatment (widely tested in ischemia-injury model), SGLT2 might represent a different therapeutic candidate because of alternative energy income pathways involved[60]. A comparison between the metabolomic changes due to SGLT2i molecules as compared to glucocorticoids is available in Figure 2.

Because use of ketone bodies depends on the targeted organ, heart as well as kidneys may be those which benefit the most from an increase in 3HB[57]. Furthermore, similarly to an ischemia-hypoxia setting, during incremental atrial pacing, fractional extraction of 3HB persist, with improved energy efficiency; and a lower use of free fatty acids in low oxygenation conditions prevents the formation of ROS[59].

Of note, even if data from animal studies are promising and suggest benefit regarding infarct size and recovery, opposite signals appear when focusing on ketone bodies[58,61]. A recent work reported a suppression in ketone body utilization by myocardial during ischemia, based on levels of ß-hydroxybutyrate in patient presenting chest pain in a retrospective population[62]. Animal models with low-carbohydrate diet inducing mild nutritional ketosis showed a worse recovery and survival, more arrythmias after induced ischemia[63,64]. However, these contradictory results, well summarized in Kolwicz and al. review[65], only raise the need for additional studies at the metabolic level.

Inhibition of the NHE

SGLT2i were also associated with the inhibition of the NHE in myocardial cells[66]. We previously described the role of NHE in the homeostasis of ischemic cells, which induce oxidative stress with elevated cytosolic Na^+ and increased mitochondrial formation of ROS through a final intracellular calcium overload. The counterbalance of such mechanism requires the regeneration of antioxidative enzymes by mitochondria, relying on NADPH, indirectly produced by the Krebs cycle, in turn activated by intramitochondrial calcium[67]. NHE inhibitors were associated with cardioprotective features in animal models of acute myocardial infarction[68]. Moreover, a chronic inhibition of NHE was associated with improvement against cardiomyocyte injury, remodeling, and systolic dysfunction[69].

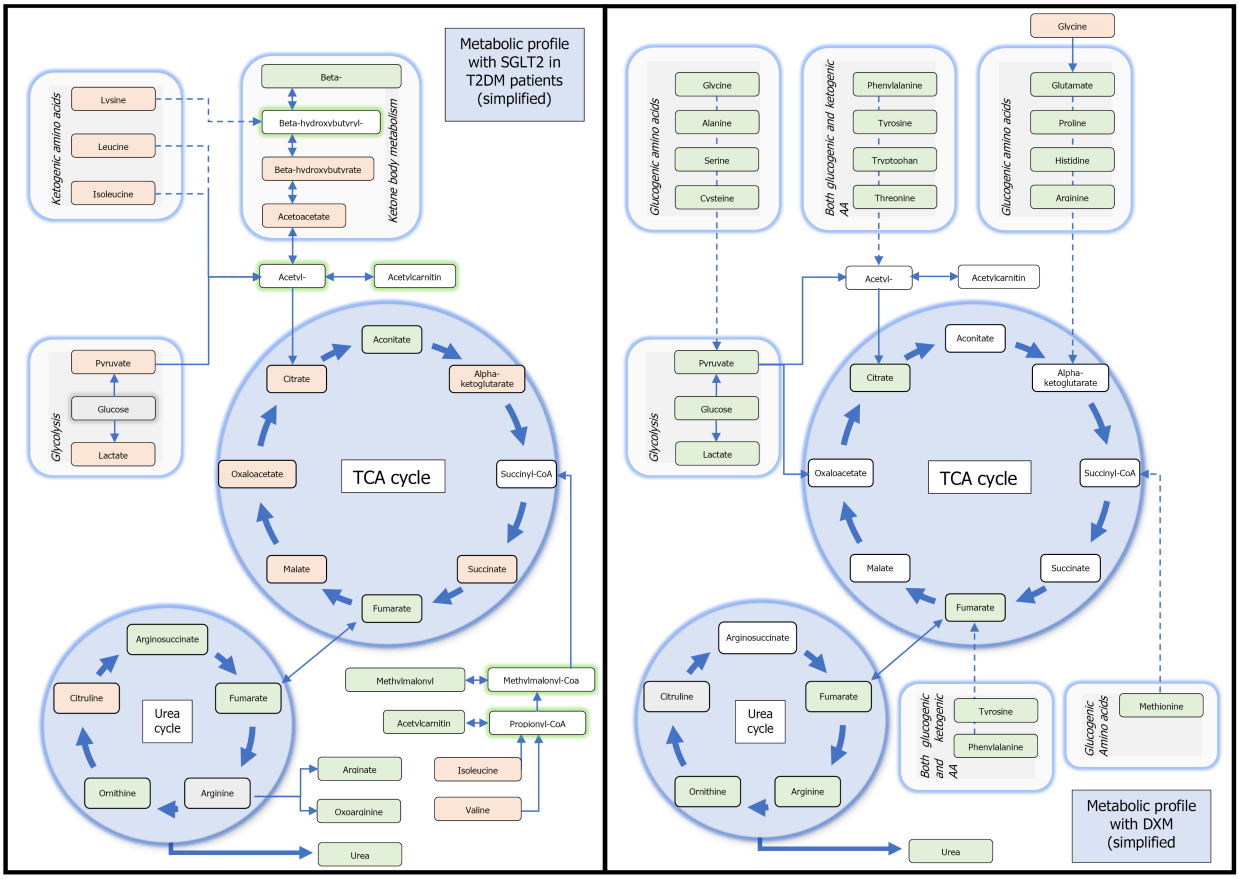


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Figure 2 Simplified comparison between metabolic profiles with sodium-glucose cotransporter-2 inhibitors or dexamethasone. Metabolites with observed high serum levels appear in light green, metabolites with supposed increased serum levels appear in highlight green with white center, those with decreased serum levels in gray center, those with unchanged serum levels appear in light orange and finally, those which remain untested appear in white. Incomes with sodium-glucose cotransporter-2 inhibitors suggest utilization of Ketone bodies and ketogenic amino acids as reactive for Krebs cycle, and indirectly urea cycle, when utilization of glucose is decreased. On the other hand, administration of dexamethasone is associated with elevated rates of glucogenic amino acids or ketogenic-glucogenic amino acids, concurring to Krebs cycle and urea cycle activations. TCA: Tricarboxylic acid cycle; DXM: Dexamethasone.

Remarkably, SGLT2i indirectly interacts with NHE. In mice, empagliflozin reversed the effects of ouabain (an agent increasing intracellular sodium)[70]. Moreover, this effect was independent from SGLT2 and indirectly caused a decreased activation of the Na^+/Ca^{2+} exchanger. The same results were observed with other SGLT2i (dapagliflozin, canagliflozin)[66].

This inhibition with empagliflozin was associated with lower rates of tumor necrosis factor alpha (TNF-α), attesting of a cell preservation and lowered inflammation through NHE inhibition.

Additional mechanisms which were hypothesized include: improved AMPK activation in myocytes [71], and cardio-fibroblasts[72]. In contrast, another study showed that concrete benefit on AMPK-pathway with SGLT2 in human cells and mouse cells *in vitro* seems unlikely because activation appeared with concentrations corresponding to the peak plasma concentrations of therapeutic doses [73].

In human cells, NHE inhibition showed similar results in atrial and ventricular myocytes, as compared to that of mice ventricular myocytes. Heart failure and atrial fibrillation were associated with increased NHE expression[74]. Finally, in human coronary endothelial cells, empagliflozin was associated with a similar reduction of oxidative stress supporting the previous hypothesis[75].

Positive effects of inhibition of NHE are not limited to better myocardial function, ionic homeostasis, or reduction of myocyte ischemic inflammation. Empagliflozin and canagliflozin in short-term treatment enhanced coronary vasodilation through NHE inhibition[66], whereas dapagliflozin needed a more prolonged treatment to reach comparable effect[76]. However, in cases of acute inflammation, a non-specific vasodilatation may occur, making it difficult to interpret supposed effect of inhibition of SGLT2[77].

Prevention of arrythmia and sudden death in ischemia-reperfusion injury

Sudden deaths and ventricular arrythmias may occur after acute ischemia and reperfusion events, and SGLT2i were associated with fewer such events. Yet, because SGLT2i do not inherently feature anti-arrhythmic properties, several mechanisms have been hypothesized[78]. An improved ionic homeo-

stasis through NHE inhibition has been suggested in the DAPA-HF trial, where 5.9% of the subjects assigned to the dapagliflozin group experimented serious rhythmic event (sudden death, cardiac arrest, ventricular arrythmias), with 7.4% in the placebo group[11]. In animal models, pre-treatment with empagliflozin reduced the incidence of reperfusion-induced ventricular arrythmia after an ischemia/reperfusion event, with the participation of the ERK1/2 pathway, involved in the RISK reperfusion-signaling pathway[79].

Role of the autonomous nervous system has also been investigated. In 2020, effects of empagliflozin *vs* placebo on cardiac sympathetic activity in acute myocardial infarction patients with T2DM (EMBODY Trial) compared empagliflozin with placebo for various electrocardiographic parameters. Heart rate variability, heart rate turbulence and electrocardiographic variations were recorded after acute myocardial infarction. Authors aimed to assess the variables associated with lethal ventricular arrhythmias. With a 6-mo-follow-up, a difference was observed between the two groups regarding sympathetic and parasympathetic stimulation[80]. Of note, to date, no study described these elements in the first few hours after an ischemic event index.

Finally, in a recent meta-analysis which analyzed the effects of SGLT2i on atrial arrythmia, sudden death and ventricular arrythmia which included 34 trials in patients with diabetes, use of SGLT2i were protective towards atrial arrythmia and sudden cardiac death, albeit several limitations existed[81].

Even if ionic homeostasis is the main hypothesis for the observed data, a plausible mechanism concurring to these results may lie on inhibition of platelet function, and antithrombin generation observed with SGLT2i. Unbalanced platelet activation and coagulation disturbance have been described during ischemic stress and associated with arrhythmia. SGLT2i have recently been associated with antiplatelet and antithrombotic features. Empagliflozin and dapagliflozin partially reduced the effects of stearic acid, an inflammatory agent inducing oxidative stress and impaired endothelial repair processes. As a result, platelets were less activated, in addition to that of ADP inhibition[82]. In male mice with T2DM model, administration of dapagliflozin showed a decreased activation and recruitment with an improved thrombin-platelet-mediated inflammation profile *in vivo* and less activated platelet with thrombin stimulation or CRP. Prolonged treatment did not affect hemostasis suggesting safety of utilization[83]. Gliflozin *via* NHE inhibition participate to maintain endothelial function[84] and endothelial production of NO. In a recent study, pharmacological analysis *in vitro* suggested that the gliflozin's antiplatelet activity synergize with NO and prostacyclin[85]. Substantial evidence sustaining an intricated mechanism.

Taken altogether, these elements encourage to explore concrete platelet and hemostasis parameters with SGLT2i in ischemic situation, to sustain a potential benefit in ischemic-reperfusion context.

EXPERIMENTAL MODELS

Models of myocardial ischemia-reperfusion

Beyond the theoretical data and focused exploratory clinical investigations, many animal models have been developed to assess the benefits of SGLT2 inhibitors in ischemia-reperfusion.

Acute administration of canagliflozin in male rat models of myocardial infarction showed decrease in infarct size, improved left ventricular systolic and diastolic function during and after ischemia, and decreased ROS[86]. Similar results were obtained with dapagliflozin[61], and the delay before the first ventricular arrythmia was lower when treated by SGLT2i. An improved communication between cardiac cells with preserved phosphorylation of gap junction protein connexin-43 was suggested[87,88]. Empagliflozin also showed similar results: reduced infarction size, better ventricular parameters, reduced systemic inflammation and ROS production, in acute or chronic administration[89,90]. The role of STAT3 phosphorylation was observed in several models[89-91]. Even if the beneficial mechanism is not yet fully determined, acute lowering of the blood glucose might be one of the potential hypothesis [92]. Interestingly, dipeptidyl peptidase 4 inhibitors were also compared to SGLT2i in murine models: SGLT2i showed greater efficacy than dipeptidyl peptidase 4 inhibitors to improve metabolic impairments and left ventricular function[93].

Recently, 16 independent animal models experiments which compared SGLT2i to control, and included 224 subjects overall, were summarized in a recent meta-analysis[94]. Regardless of diabetes, SGLT2is were significantly associated with fewer myocardial ischemia-reperfusion injuries and infarct size. Additionally, systemic treatment performed better than local administration, and longer-term treatment was associated with better results.

Other organ models

On top of myocardial protection, other organs have been tested.

In a model of lung injury due to ischemia-reperfusion, empagliflozin was tested on respiratory function, tissular and cellular analyses. Similarly, as in cardiac usage, SGLT2i was associated with lower levels of circulating cytokines in bronchoalveolar liquids, those were dependent on improved phosphorylation of pulmonary ERK1/2[95].

In models of ischemia-reperfusion-induced kidney injury, dapagliflozin was associated decreased biomarkers of renal failure (blood urea and creatinine) and fewer tubular injuries. Furthermore, under hypoxic condition, dapagliflozin reversed cellular death. Similarly, as in heart and lung, phosphorylation of AMPK and ERK1/2 was improved[96]. Remarkably, similar observations were made in non-diabetic rats[97].

Finally, in neurons, SGLT2i may interact with SGLT2 and SGLT1, expressed in human center nervous system[98]. Similar ischemia-reperfusion injuries may be performed in neurons, and empagliflozin in was associated with smaller infarct size and improved neuronal functions than in control rats. The main pathway studied was the HIF-1α/VEGF cascade, on which suppression of neuronal expression of Caspase-3 by empagliflozin had positive neuronal effects[99]. Moreover, role of Caspase-3 repression in hyperglycemic rats suggested an association between empagliflozin use and a decrease in TNF-α[100].

CONCLUSION

Beyond the cardiovascular benefits observed in patients with chronic heart failure treated by SGLT2i, data from large clinical trials including EMPA-REG or DAPA-HF may suggest a benefit through ischemia-reperfusion events. The inhibition of the NHE may play a pivotal role in such cardioprotective feature and further investigations towards the immunomodulatory properties of SGLT2i drug-class are warranted.

FOOTNOTES

Author contributions: Quentin V and Singh M co-wrote the manuscript, Nguyen LS supervised the study and provided critical reviewing.

Conflict-of-interest statement: None.

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 696-716

DOI: 10.4239/wjd.v13.i9.696

ISSN 1948-9358 (online)

REVIEW

Evolving spectrum of diabetic wound: Mechanistic insights and therapeutic targets

Raja Chakraborty, Pobitra Borah, Partha Pratim Dutta, Saikat Sen

Specialty type: Medicine, Research and Experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Samadi N, Iran; Wu QN, China

Received: May 1, 2022
Peer-review started: May 1, 2022
First decision: May 29, 2022
Revised: June 12, 2022
Accepted: August 15, 2022
Article in press: August 15, 2022
Published online: September 15, 2022

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Abstract

Diabetes mellitus is a chronic metabolic disorder resulting in an increased blood glucose level and prolonged hyperglycemia, causes long term health consequences. Chronic wound is frequently occurring in diabetes patients due to compromised wound healing capability. Management of wounds in diabetic patients remains a clinical challenge despite many advancements in the field of science and technology. Increasing evidence indicates that alteration of the biochemical milieu resulting from alteration in inflammatory cytokines and matrix metalloproteinase, decrease in fibroblast and keratinocyte functioning, neuropathy, altered leukocyte functioning, infection, *etc.*, plays a significant role in impaired wound healing in diabetic people. Apart from the current pharmacotherapy, different other approaches like the use of conventional drugs, antidiabetic medication, antibiotics, debridement, offloading, platelet-rich plasma, growth factor, oxygen therapy, negative pressure wound therapy, low-level laser, extracorporeal shock wave bioengineered substitute can be considered in the management of diabetic wounds. Drugs/therapeutic strategy that induce angiogenesis and collagen synthesis, inhibition of MMPs, reduction of oxidative stress, controlling hyperglycemia, increase growth factors, regulate inflammatory cytokines, cause NO induction, induce fibroblast and keratinocyte proliferation, control microbial infections are considered important in controlling diabetic wound. Further, medicinal plants and/or phytoconstituents also offer a viable alternative in the treatment of diabetic wound. The focus of the present review is to highlight the molecular and cellular mechanisms, and discuss the drug targets and treatment strategies involved in the diabetic wound.

Key Words: Diabetic Wound; Diabetic Foot Ulcer; Epigenetic mechanisms; Therapeutic agents; Molecular Targets; Phytoconstituents

Core Tip: This paper reviewed molecular pathways and epigenetic mechanisms involved in the pathogenesis of diabetic wounds. The role of microbiota, oxidative stress, inflammatory cytokines, and alteration in the factors involved in normal wound healing process was highlighted. Molecular targets of therapeutic agents, the role of phytochemicals was discussed. The efficacy of several pharmacotherapy, treatment strategies, and recent clinical trials aiming to improve the outcome of diabetic foot ulcers was reviewed.

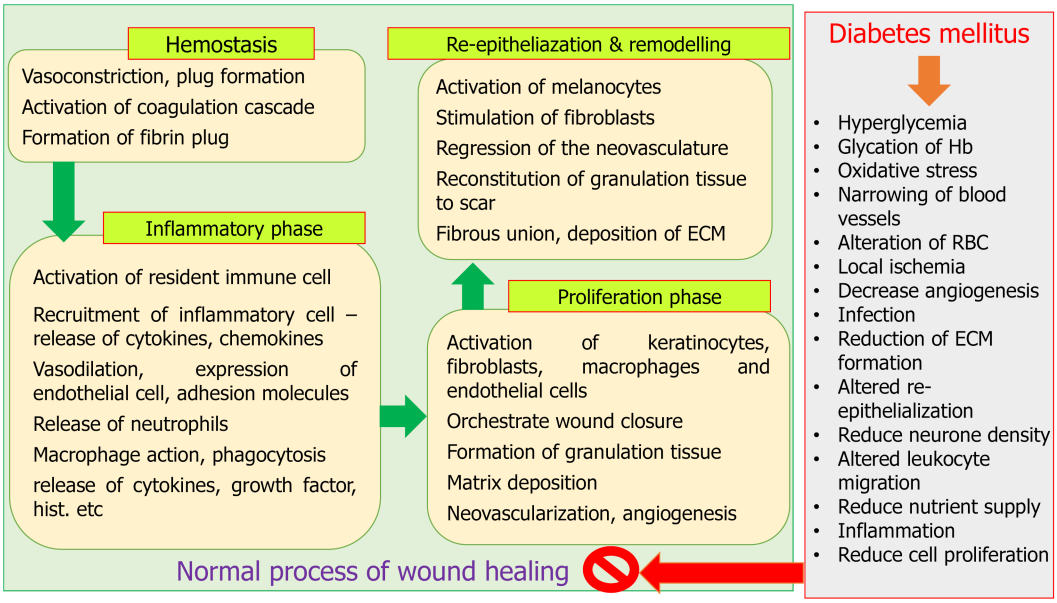
Citation: Chakraborty R, Borah P, Dutta PP, Sen S. Evolving spectrum of diabetic wound: Mechanistic insights and therapeutic targets. *World J Diabetes* 2022; 13(9): 696-716
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/696.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.696

INTRODUCTION

The process of wound healing is complex and requires spatial as well as temporal synchronization between different types of cells with specific functions. Hemostasis, inflammatory phase, proliferative phase, re-epithelialization, and remodelling phase are the four major phases of wound healing process, which result in the restoration of functional integrity of tissues[1-3]. Alteration in the microenvironment due to diabetes mellitus (DM) results in a change in the level of oxygen, chemokines, synthesis of growth factors, extracellular matrix, oxidative stress that in turn alter normal cellular recruitment and activation, and induce impaired or delayed wound healing[1,3]. Ageing, genetic disorders, obesity, and metabolic disorders including DM, are responsible for the abnormal wound healing process, that enhances the risk of developing chronic wound[1,3,4]. It was estimated that chronic wounds directly affect the quality of life of about 2.5% population of the United States, and the medicare cost for management of all wounds and the related situation was projected between $28.1 to $96.8 billion[5]. Management of wounds in the diabetic individual is a major clinical and social concern. The hyperglycaemic environment in diabetic people causes impaired and delayed wound healing[4], making the situation more perilous as the number of diabetic people is increasing day by day. It was also found that treatment and management of diabetic ulcers and surgical wounds were the most expensive[5]. The IDF Diabetes Atlas estimated that the prevalence of DM in 2021 was 10.5% (536.6 million people in the 20-79 year age group), which will increase to 12.2% (783.2 million in the 20-79 year age group) by 2045. Global health expenditures related to the management of DM and its complications are expected to reach $966 billion in 2021[6]. It was also predicted that almost half of the adult population (44.7%; 239.7 million of 20-79 years old) were unaware of their diabetic condition. People may develop micro and macrovascular complications during an asymptomatic diabetic state[7]. Impaired or delayed wound healing affects about 25% of diabetic people. A study suggested that 1 in 3 to 1 in 5 diabetic individuals are at risk of chronic non-healing wounds, including diabetic foot (with a very high recurrence rate) in their lifetime[8]. Zhang *et al*[9] estimated that the global prevalence of diabetic foot is 6.3%, which usually affects type 2 diabetic people, older people, and people with a longer duration of DM.

Both the extrinsic and intrinsic factors are responsible for delay in the wound healing process in diabetic patients. Repeated trauma or mechanical stress in the diabetic foot can lead to neuropathy and ischemic situation. Glucose-rich environment results in increased generation of advanced glycation end-products (AGEs) and elevated levels of inflammatory cytokines [*i.e.*, interleukin (IL)-1β and tumor necrosis factor α (TNF-α)] for a persistent period that hinders the normal process of wound healing[10,11]. In turn, hyperglycemia reduces collagen synthesis, growth factor production, macrophage function, angiogenic response, migration and proliferation of fibroblast and keratinocyte, epidermal nerve count, and the balance between extracellular matrix (ECM) component accumulation and matrix metalloproteinase (MMP) induced remodelling [4,12]. The normal wound healing process and effect of the hyperglycemic condition are depicted in Figure 1.

Despite the presence of protocols to standardize care in the diabetic wounds, as well as numerous advancements in scientific research and in clinical fronts, DM remains a problematic situation for wound healing. This paper is an attempt to highlight the mechanistic insights, plausible therapeutic targets, and pharmacotherapeutic approaches, particularly the role of phytochemicals in the management of diabetic wounds, in light of recent shreds of evidence.



DOI: 10.4239/wjd.v13.i9.696

Figure 1 Normal process of wound healing and effect of diabetes mellitus. ECM: Extracellular matrix.

MECHANISTIC INSIGHTS OF DIABETIC WOUND

Wound healing, being an evolutionary conservation process, restores impaired epithelial barriers through a cascade of events including inflammatory responses, proliferation, cellular migration, angiogenesis, and remodeling of tissues[13]. DM interferes with the normal healing process to provoke non-healing wound, and leads to complications including walking difficulty and infections like septicemia, abscess, cellulitis, osteomyelitis, and gangrene[4]. Acute diabetic wounds with an impaired healing process of unknown aetiology are the first signs of chronic diabetic wounds. Hyperglycemia, hypoxia, chronic inflammation, neuropathy, circulatory dysfunctions, alteration in neuropeptide signalling, and infections impede the diabetic wound healing process. Importantly, due to the heterogeneous nature of the diabetic wound, there exist no clear implications from the pathogenic vantage. The following subsections discuss the potential factors underlying the pathogenesis of diabetic wound.

Molecular implications

Impaired wound healing associated with diabetes remains inconclusive. However, the alterations in cellular factors and biochemical mediators have been believed to be involved in the development and progression of diabetic wound (Figure 2). Factors like hyperglycemia and oxidative stress in diabetic patients result in dysregulated macrophage polarization through modulation of epigenetic codes to delay the process of wound healing[14,15]. Hyperglycemia is implicated in impaired wound closure in diabetic foot ulcers (DFUs), with reduced skin cell function and the formation of atherosclerosis and neuropathy as possible contributors[8]. The development of atherosclerosis leads to alteration in the physiology of endothelial cells along with deprivation of nutrients in the wound site, critically affecting the healing process. Patients with type 1 DM are more prone to macrovascular diseases, especially affecting femoral and metatarsal arteries[16,17]. DM associated early microvascular deficiencies include decreased capillary size, basement membrane thickening, and arteriolar hyalinosis. Thickening of the membrane disrupts the physiological exchanges and causes altered leucocyte migration, and thereby increasing the risk of microbial infections[18,19]. Hyperglycemia disrupts protein translation as well as the migration and proliferation of fibroblasts and keratinocytes involved in the process of re-epithelialization[20-22]. For instance, altered expression of proteins like cytoskeletal keratin proteins (K2/K6/K10) associated with keratinocyte differentiation, and LM-3A32, a laminin-5 α3 chain precursor protein that regulates epithelial cell binding to the basement membrane, was reported in subjects with DFUs[23]. As LM-3A32 is required for the survival and differentiation of keratinocytes, reduction in this protein affects the re-epithelialization process[24]. Interestingly, the expression of mRNA and microRNA was found to be non-significant in diabetic and non-diabetic foot skin fibroblasts[25]. However, fibroblasts from DFUs were reported to exhibit altered morphology, growth factor unresponsiveness, ECM deposition, and reduced proliferation and migration[26-29]. Impaired vasculogenesis and angiogenesis due to deregulation of the growth factors and receptors leads to impaired wound healing. Dysfunctional endothelial progenitor cells (EPC) or reduction in their numbers and transition to proinflammatory EPC phenotypes have been implicated in DM[30,31]. Notably, EPC dysfunction and altered recruitment are contributed by hyperglycemia, chronic inflammation, oxidative

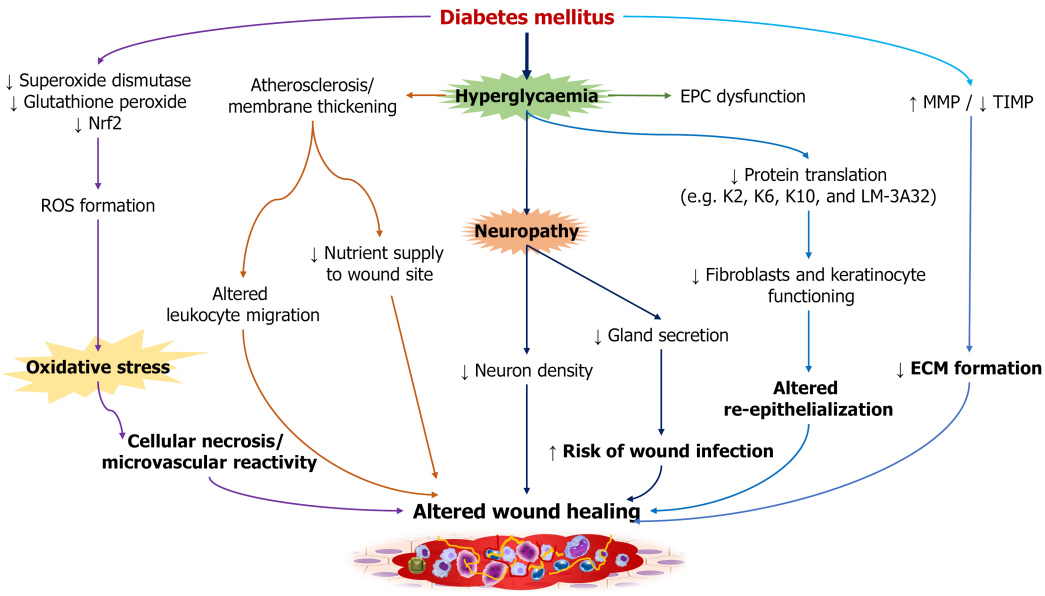


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Figure 2 Altered cellular factors and biochemical mediators involved in the development of diabetic wound. Nrf2: Nuclear factor erythroid factor 2-related factor 2; ROS: Reactive oxygen species; EPC: Endothelial progenitor cell; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; ECM: Extracellular matrix.

stress, and activation of NADPH oxidase associated with diabetes pathogenesis[32]. Prolonged IL-1β production and reduced expression of peroxisome proliferator-activated receptor-γ are also involved in the impairment of wound healing in DM[33]. Higher expression of MMPs including MMP-1, 2, 8, 9, 14, and 26, and lower expression of their tissue inhibitors were also reported in diabetes[34,35]. In patients with DFUs, it was reported that there is an increase in MMPs and a decrease in tissue inhibitor of metalloproteinase (TIMP)-2, which supports the fact that in a proteolytic environment, diabetic wound fails to heal due to reduction in ECM formation[36]. An increase in MMPs also contributes to matrix degradation, delayed cell migration, and inhibition of collagen deposition[36]. Oxidative stress resulting due to decrease action of enzymes like superoxide dismutase and glutathione peroxidase augment diabetic wound. Overproduction of reactive oxygen species (ROS) from hexosamine, polyol, and AGE pathways affects the later stages of diabetic wound healing, particularly by damaging the peripheral nerves. Consequently, the detrimental effect on the structure, supply, and metabolism of peripheral nerves (neuropathy) increases the risk of DFU development[37]. Hyperglycemia not only contributes to impaired healing but also makes skin prone to injury. Decrease in nuclear factor erythroid factor 2-related factor 2 (Nrf2) in diabetic patients increases the oxidized proteins and ROS generation[38,39]. In Nrf2 knockout mice, delayed wound healing was reported when compared to a $Nrf2^{+/+}$ mice, possibly due to oxidative DNA damage, elevated MMP-9 expression, and lower level of transforming growth factor-beta 1 (TGF-β1) expression[40]. Hyperglycemia also leads to an increase in neutrophil extracellular traps release, which has been implicated in delayed wound healing in both murine and human models[13,41].

The formation of a diabetic wound is also linked to several forms of neuropathy, including sensory, motor, and autonomic. Sensory deficits, for example, cause a loss of protective symptoms, whereas motor neuropathy causes anatomical deformities and ischemic death in the plantar region of the foot [42], and autonomic neuropathy reduces sweat secretion from the glands, making skin dry and increasing the risk of infection and pruritus[43]. Furthermore, neuropathy causes a decrease in neuron density, causing impairment in wound healing. Primarily, diabetic neuropathy occurs in nerves that are dependent on nerve growth factor[8]. Impaired microvascular processes along with autonomic neuropathy and denervation of sympathetic nerves disrupt the blood flow. Cellular necrosis and microvascular reactivity are also triggered by Poly (ADP-ribose) polymerase enzyme *via* oxidative DNA damage[16,44]. In diabetic neuropathy, impairment of C-fibre dependent neurovascular responses leads to abnormality in the release of histamine, substance P, and calcitonin related peptide, thus exhibiting altered vasodilatation in case of stress like pressure or trauma[44]. However, the direct link between neuropathic abnormalities and glucose control is yet to be proven.

Epigenetic mechanisms

Despite the fact that epigenetic pathways have a role in a variety of diabetic complications, evidence of epigenetics in diabetic wound or impaired wound healing is still emerging. The role of microRNA in diabetic wound was first highlighted by a study that revealed up-regulation of miR-503 in plasma

obtained from DFUs and in human umbilical vein endothelial cells (HUVEC). *In vitro* experiment suggested the detrimental effect of forced miR-503 expression on function of HUVEC cells resulting due to impaired migration, proliferation, and formation of blood vessels[45]. Blockage of miR-503 expression showed improvement in angiogenesis in diabetic animals with limb ischemia[45,46]. Another study demonstrated that diabetic mice had a distinct microRNA signature, with differential expression of fourteen microRNAs[47]. Of these, expression of miR-146b was found to be up-regulated by 30 fold. Though miR-21 was up-regulated in diabetic skin, it was reduced in diabetic wound healing. This study suggested the necessity of miR-21 expression for fibroblast migration and miR-21 knock-down results in altered cellular migration. Similarly, another study showed the stabilisation of hypoxia-inducible factor α leads to miR210 expression, which silences the expression of E2F3, an important element of wound healing[46]. This implies the fact that epigenetic changes in miR-210 lead to impaired wound re-epithelialization and reduced proliferation. The involvement of DNA methylation in impaired diabetic wound healing is being studied in many experiments. A study reported inhibition of DNA methyltransferase 1 (DNMT1), an enzyme that transfers a methyl group to the cytosine ring to produce 5-methylcytosine associated with transcriptional repression, suppresses inflammatory signals in bone marrow derived macrophages and also promotes M2-like macrophage formation[48]. This finding was supported by DNMT1 knockdown in db/db mice showing improvement in wound healing[49]. In contrast to that, demethylation of MMP-9 promoter in keratinocytes was reported to be involved in the induction of diabetic wound[50,51]. Apart from the role of DNA methylation in wound healing, it is also associated with metabolic memory, insulin resistance, and other diabetic complications[52,53]. Unlike DNA methylation, histone methylation does not always lead to transcriptional repression. Instead, it silences or promotes transcription based on the target residue and methyl groups. Methylation of histone H3K4 is regulated by SET domain containing protein family, particularly MLL1 that promotes inflammatory gene expression in nuclear factor kappa B-dependent manner[54-56]. A study reported the role of mixed-lineage leukemia-1 (MLL1) is to catalyze H3K4me3 deposition in macrophages during the process of wound healing[57]. Delayed wound healing and reduced pro-inflammatory cytokine generation were reported in a myeloid-specific MLL1 deletion in mice. Monocytes isolated from patients with type-2 DM demonstrated higher MLL1 expression, indicating dynamic regulation of MLL1 expression during diabetic wound healing. In diet-induced obesity model of diabetes, increased expression of histone demethylase (*i.e.*, lysine-specific demethylase 6B or JMJD3) that targets H3K27me3 was seen in wound macrophages[58,59]. Epigenetic regulation of IL-6 expression in neutrophils was believed to be impacted by toll-like receptor (TLR) activation associated with increased H3K27ac, H3K4me3, and acetylated histone H4[60]. Re-epithelialization is also promoted by JMJD3 expression, which induces keratinocyte migration to the wound site[61]. Similar report suggests upregulation of JMJD3 expression on the wound site is necessary for early onset of the wound healing process, which is absent in diabetic wound[62]. However, it will be too oversimplified interpolation as most of these findings are limited to normal wounds; thus, further studies are warranted. Other histone modifications including histone acetylation or deacetylation, histone phosphorylation, histone-arginine demethylation or methylation, and ATP-dependent chromatin remodelling are also being studied for their potential role in diabetic wound healing[62].

Microbiological perspectives

Certainly, pathogenic infections are not directly associated with the pathophysiology of diabetic wounds but are critical from the vantage of impaired wound healing, hospitalization, morbidity, and amputation. However, the role in the initiation of diabetic wound in case of trauma remains unclear. The rapid spreading of infections and high microbial burden exhibit detrimental effect on the wound healing process. Injury to the superficial skin layer allows polymicrobial contamination and colonization, affecting diabetic wound. Particularly, infections like cellulitis, osteomyelitis, and abscesses are of major concern[16]. The advent of high-throughput sequencing technologies like microarray, 16S rRNA sequencing, and whole-genome sequencing have enabled the expansion of diabetic wound microbiome. Diabetic wound has demonstrated higher colonization of *Staphylococcus aureus* and *S. epidermidis*[63]. Another study reported *Staphylococcus*, *Enterobacteriaceae*, and *Pseudomonas* to be the most common colonizers in DFUs[64]. Stratification of DFUs as per infection severity revealed higher bacterial diversity in severely infected DFUs, while *Staphylococcus* and *Streptococcus* were found to be the most abundant in mild-to-moderate DFUs[65]. It was similar to a previous finding that found diverse microbiota (with higher incidence of anaerobic and Proteobacteria infection) in deep chronic ulcers, while *Staphylococcus* was found to be abundant in acute and superficial ulcers[66]. However, contrary to these reports, another study reported *Staphylococcus* spp. to be the primary culture detected in the microbiome in diabetic foot osteomyelitis[67]. A higher prevalence of *S. aureus* colonization in DFUs and intact diabetic skin lead to systemic infection and osteomyelitis[63]. Indeed, in a Nigerian observational multi-center study, it was reported that the presence of osteomyelitis is an important predictor of wound healing in hospitalized patients with DFUs[68]. The expression of proteolytic factors by *Streptococcus* and *Staphylococcus* are believed to be the disruptor of skin barrier. For example, SpeB released by *Streptococcus* leads to cleavage of desmoglein 1 and 3 that causes epidermal barrier damage [69]. The alkaline environment of DFUs contributes to the formation of bacterial biofilm, leading to complex host–microbiome interaction[70]. The formation of bacterial biofilm along with alkaline pH

affects drug action and is responsible for antibiotic resistance[71]. One should appreciate various other factors influencing this intricate microbiome network and its potential correlation with clinical significance. Keeping all this evidence in sight, more longitudinal studies are anticipated for an adequate understanding of the probable relevance of the microbiome to clinical outcomes.

PLAUSIBLE DRUG TARGETS

In recent studies, neuropathy, peripheral vascular disease, and impaired wound healing have all been identified as key contributors to diabetic wounds and have all become critical targets in improving wound healing in diabetic patients. But despite of this well-established knowledge, comparatively fewer treatment options are being implemented in routine practice. The expression of cellular components of participating cells, as well as cytokines, growth factors, and other molecular factors required for coordinating the normal healing process, is impaired in diabetic wounds, and as a result, they are unable to progress in synchrony and are primarily checked in the inflammatory phase. Therapeutic strategy targeting such cellular and molecular pathway could be useful for effective management of diabetic wound.

As a result of elevated blood sugar levels in diabetic conditions, damage to nerve fiber occurs, leading to diabetic neuropathy, which can be sensory, motor or autonomic[72]. Sensory neuropathy can result in one of two outcomes: a painful foot or a foot that is devoid of sensation[12]. Motor neuropathy is accompanied by muscle weakening, atrophy, and paresis. The inability of an intrinsic muscle to keep the foot in its normal state resulting because of weakening of inter-osseous muscles in the foot, which contributes to foot deformity. When the foot deforms, the pressure distribution throughout the foot changes, and aberrant pressure develops at various locations on the foot[73]. Keratosis and callus development occur as a result of repeated pressure, which leads to damage to callused areas and induces ulcer formation beneath the callus that further causes cracks on the foot[74-77]. Malfunctioning of the sympathetic nerves supplying the sweat glands in the foot reduces the sweat and moisture in the feet, which leads to the development of cracks[78]. Inflammation, necrosis, and ulceration result when an unnoticed injury is combined[16,79]. Currently, duloxetine, anticonvulsant pregabalin and opioid tapentadol are being prescribed for diabetic peripheral neuropathy. Besides these, a substance like α-lipoic acid has shown effectiveness in delaying or reversing peripheral diabetic neuropathy through its multiple antioxidant properties[80]. Neuropeptides such as substance-P and neuropeptide-Y are also been found to be effective in diabetic neuropathy and associated wound[81,82].

Peripheral vascular dysfunction is another major cause of diabetic wound in a majority of diabetic patients. The wound healing process in the diabetic condition is hampered by the altered physiological response due to glycation of hemoglobin, alteration of the red blood cell membrane and narrowing of blood vessels which cause the decreased supply of nutrients and oxygen to tissues[12]. The development of atherosclerotic plaque in diabetic patients also leads to the development of non-healing wound. Therefore, pharmacotherapeutic agents like antioxidant phytochemicals that can avert oxidative stress and formation of AGEs could be useful in treating diabetic wound.

Bacterial infection is one of the most common causes of wounds, and diabetic individuals are more vulnerable to it because of delayed wound healing and immunosuppression[83,84]. The biofilms created by microorganisms protect them from antimicrobial agents and the immune system while also interfering with the healing process, which is one of the most prevalent reasons for amputation of lower limbs in diabetic wounds[14]. Therefore, empiric therapy should include broad-spectrum antibiotics. In recent times, drug resistance is a bigger problem and several drugs are in use in the treatment of DFU.

Neutrophils, monocytes, macrophages, keratinocytes, fibroblasts, T cells, B cells, mast cells, and endothelial cells are all involved in wound healing and are responsible for the formation and modulation of pro-inflammatory cytokines and growth factors such IL-1, TNF-α, IL-6, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), and TGF-β. Hyperglycemia and oxidative stress lead to dis-regulation of these cells, resulting in delayed wound healing[85-87]. Increased amounts of pro-inflammatory cytokines cause an inflammatory cascade to be disrupted, resulting in hyper-inflammation and insulin resistance. These also lead to reduced angiogenesis and microvascular issues, impaired macrophage and neutrophil function, impaired keratinocyte and fibroblast migration and proliferation, and impaired growth factor generation[15,88-90]. Many of these cells play a vital role in the immune response, which is also important for wound healing. Various chemokines whose expression can regulate the function of immune cells have the potential to enhance wound healing. Mast cells with close coordination with macrophages, endothelial cells, and fibroblasts, play a key role in matrix remodeling and disrupt the balance of pro- and anti-angiogenic molecules in wound tissues, affecting angiogenesis and vascular regression in the proliferative and remodeling phases, respectively[91-93]. As a result, mast cell degranulation inhibitors such as disodium cromoglycate, quercetin, and luteolin may be promising options for improving diabetic wound healing [92]. Heat shock proteins (HSPs) aid wound healing by attracting dermal fibroblasts, stimulating cell proliferation and keratinocyte differentiation, reducing oxidative stress, ameliorating actin microfilaments, aiding endothelial cell migration, and enhancing pro-collagen synthesis and protein

homeostasis[94,95]. Reduced levels of HSPs and their downstream components TLR4 and p38-MAPK (mitogen-activated protein kinases) in diabetic patients are responsible for the slowed healing process [96]. Therefore, targeting this as a therapeutic target could be useful in diabetic wound conditions.

Growth factors are biologically active polypeptides that play an important role in the onset and maintenance of wound healing[97]. In diabetics, any change, *i.e.*, down-regulation of growth factor receptors and rapid degradation of growth factors, causes wound healing to be delayed. Factors such as VEGF, IGF, TGF-β, KGF24, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), TNF-α, and IL-6 are significantly reduced in diabetes patients, and several of them have been demonstrated to significantly enhance wound healing in many studies. Growth factors that cause a molecular alteration in the wound micro-environment may help patients with non-responsive wounds. PDGF is a major serum mitogen that promotes fibroblast proliferation, matrix formation, and connective tissue maturation[98]. They attract fibroblasts and inflammatory cells and aid in the production of glycosaminoglycans, proteoglycans, and collagen. During the healing process, it is a critical mediator in fibroblast migration and proliferation, the formation of granulation tissue proteins and provisional extracellular matrix, and angiogenesis[99]. The expression of PDGF and its receptors is reduced in diabetic wounds, and many clinical studies employing PDGF have shown improved healing time[100,101]. It is indicated for the treatment of infections that have spread to deeper subcutaneous tissues or beyond areas with a sufficient blood supply[102]. Another growth factor, bFGF, has a stimulatory effect on fibroblast growth and differentiation, as well as the proliferation of vascular smooth muscle cells, endothelial cells, ECM metabolism, growth, and movement of mesodermally derived cells, all of which speed up the formation of granulation tissue and promote wound healing [103]. Angiogenesis, cell proliferation, migration, differentiation, neo-vascularization, re-epithelialization, and collagen disposition were all stimulated by the clinical application of bFGF, all of which contribute to wound healing[104]. It promotes mesodermal cell chemotaxis and extracellular matrix growth and expedites both acute and chronic wound healing, which gives a scar-free cure[105]. VEGF is a potent angiogenic cytokine that has a substantial impact on healing and promotes rate-limiting processes in vasculogenesis and angiogenesis[106]. Low VEGF levels cause impaired wound healing and aberrant VEGF receptor patterns in diabetics. Decreased VEGF mRNA levels, increased VEGF receptor (VEGFR)-1 Levels, and decreased VEGFR-2 Level are some of the key causes of wound non-healing[107]. In diabetic wounds, VEGF leads to an increase in capillary density, which enhances blood perfusion and metabolism in the wounded tissue[108]. VEGF causes an increase in capillary density, which improves blood perfusion and metabolism in the wounded tissue. This leads to the facilitation of the supply of oxygen and nutrients to assist the growth and function of reparative cells. It is the primary regulator of wound revascularization and permeability and participates in the formation of granulation tissue. On binding with the EGF receptors, EGF causes an increase in epidermal cell, cell motility, cellular migration, mesenchymal regeneration, angiogenesis and cell proliferation[109]. Application of EGF into the wound site results in a greater pharmacodynamic response in terms of granulation tissue growth and wound closure. IGF-1 promotes wound healing by assisting in cell granulation and re-epithelisation, promoting endothelial cell chemotaxis and keratinocyte and fibroblast proliferation, while lower levels of both IGF-1 and TGF-β in wound tissue cause wound healing to be delayed[110, 111]. TGF-β attracts and stimulates inflammatory cells such as neutrophils, macrophages, lymphocytes, keratinocytes, and fibroblasts, as well as the synthesis of growth factors, which speed up vascularisation, angiogenesis, and ECM synthesis while slowing down ECM degradation[112]. Therapeutic agents or strategy, which can ameliorate this positively, are useful in diabetic wound condition. Several drugs and phytoconstituents have shown their positive effect in diabetic wounds targeting these biomolecules. The use of platelet-rich plasma (PRP), EGF, PDGF, and FGF has shown promising effects in the treatment of diabetic wound in a better way.

MMPs are a class of endopeptidase that play a key part in wound debridement, as well as angiogenesis, epithelialization, and extracellular matrix remodeling[113]. Matrix proteins such as collagens, basement membrane collagens, proteoglycans, elastin, and fibronectin are digested by the MMPs. TIMPs form a complex with MMPs, limiting interaction with the active site. A balance between MMPs and TIMPs is required for wound healing, which is impaired in diabetes patients[114]. Increased protease activity caused by high MMP levels in diabetic wounds causes tissue damage and slows down normal repair processes[115]. This is due to altered MMP expression and decreased TIMP expression in diabetic conditions, which results in high levels of pro-inflammatory and pro-fibrotic cytokines due to increased inflammatory cell activation and invasion, and indirectly affects MMPs through the formation of advanced glycation products which leads to the loss of growth factors, receptors, and matrix proteins essential for wound healing[111,114]. Drugs, therapy that is useful in diabetic wounds are found to act by enhancing collagen synthesis, decreasing inflammatory cytokines, AGEs *etc*. Further, identifying the therapeutic agents that can inhibit MMPs, *i.e.*, MMP-1, MMP-8, and MMP-9 could be important in the management of diabetic wounds.

Autologous stem cells capable of self-renewal and multi-lineage differentiation have been used in diabetic wound. Clinically, bone marrow-derived mononuclear cells and mesenchymal stem cells are the most successful stem cell therapies[116,117]. Besides these, several other targets to promote diabetic wound healing include stimulation of nitric oxide production and up-regulation of endothelial NO synthase and nitric oxide (NO) expression[118-120], a decrease of AGE receptors[121], collagen

generation and epitheliazation *etc*[122-124]. Figure 3 represents plausible drug targets for diabetic wound.

MANAGING DFU: PHARMACOTHERAPY

Hyperglycemic environments worsen the wound situation and delay the wound healing process, thus controlling blood sugar levels is important for the wound healing process. Several antidiabetic medications [*i.e.*, insulin, metformin, sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, thiazolidinediones] found effective in controlling not only blood sugar levels but also promoting the healing of a wound. Metformin was found to be effective in wound healing, which may act by improving epidermis and deposition of collagen, and increasing TGF-β production that may link with angiogenesis process, inflammatory reaction, re-epithelization, and remodeling process. DPP-4 inhibitors may act by increasing the generation of SDF1α, which is crucial for wound repair process[11, 125]. Drugs like simvastatin (enhance VEGF and NO content at wound site) and phenytoin (enhance VEGF and FGF in wound area) have also shown some positive effects in wound healing[11,125].

Infection caused by microbes is a major problem in DFU. Antimicrobial therapy targeting Gram -ve, Gram +ve, anaerobic bacteria, and certain fungi, contributing to the pathophysiology of DFU, is also a key approach. Povidone iodine solution (10%), chlorhexidine, acetic acid (5%), and silver compounds can be used to treat the mild wound. Antibiotics like amoxicillin + clavulanate, ampicillin, dicloxacilline, cephalosporin (*i.e.*, cephalexin, cefoxitin, ceftriaxone, and ceftazidime), quinolones (*i.e.*, ciprofloxacin, levofloxacin), metronidazole, and clindamycin used to cure moderate DFU[126]. Drugs like piperacillin/tazobactam + clindamycin, meropenem, imipenem, ertapenem, ampicillin + sulbactam are used to cure severe infections caused by different Gram +ve and Gram -ve bacteria, anaerobes, MRSA, *P. aeruginosa*, while vancomycin, linezolid, and daptomycin can cure MRSA[11]. Cefepime, ceftazidime, meropenem, and piperacillin/tazobactam are recommended to use against *P. aeruginosa;* Metronidazole and clindamycin are used to manage infection caused by anaerobes, while osteomyelitis can be managed with quinolones and linezolid. Further, the effectiveness of different drugs like penicillin, cephalosporin (ceftazidime, ceftriaxone, and cefuroxime), dicloxacillin, vancomycin aztreonam, cefalotin, clindamycin, cefoxitin, gentamicin, imipenem, piperacillin/tazobactam, imipenem, metronidazole, amikacin, levofloxacin, and cefalotin against microbes contributing in DFU infection also established[126]. Dressings with hydrogels, acrylics, hydrofibers, films, hydrocolloids, calcium alginates, polyurethane foam and ciprofloxacin-loaded calcium alginate wafer are also used to control the infection[126,127].

Debridement (elimination of bacterial biofilm and necrotic tissue from wound site), offloading (complete/partial removal of pressure), PRP, EGF, PDGF, FGF, oxygen therapy, negative pressure wound therapy (NPWT), low-level laser therapy (LLLT), extracorporeal shock wave therapy (ESWT), bio-engineered skin substitutes/ soft tissue substitutes (amniotic membrane, autologous stem cell therapy, bi-layered bio-engineered skin substitute, human fibroblast-derived dermis, porcine small intestine submucosa), and maggot therapy are also used successfully to cure DFU[127-129].

In clinical practice, control of blood glucose level, periodical foot screening, patient education, use of therapeutic footware by susceptible people, prophylactic arterial revascularization are important in prevention of DFU[130]. Off-loading in different grades of DFUs (grade 1B, 1C), and diagnosis of diabetic foot osteomyelitis are recommended in grade 1B, 1C, 2C DFU using different screening methods by Society of Vascular Surgery[130]. Surgical debridement to remove necrotic and devitalized tissue, use of proper dressing to create and maintain moist environment, reducing plantar pressure and shear stress (off-loading), vascular assessment in patient with peripheral arterial disease are the vital part of wound care in diabetic people[130,131]. Microbial infection is a major concern in DFU, and in such cases, use of antimicrobial agents is advised conserving extent of infection[131]. A number of adjuvant therapy as discussed in previous section are also used for effective wound healing. Further, adequate glycemic control is important also in acceleratd healing of DFU's[130,131].

RECENT APPROACHES IN CLINICAL TRIALS

Several treatments /medicines have been successfully investigated clinically for their beneficial effects on the diabetic wound, especially in DFUs which was tabulated in Table 1[132-159].

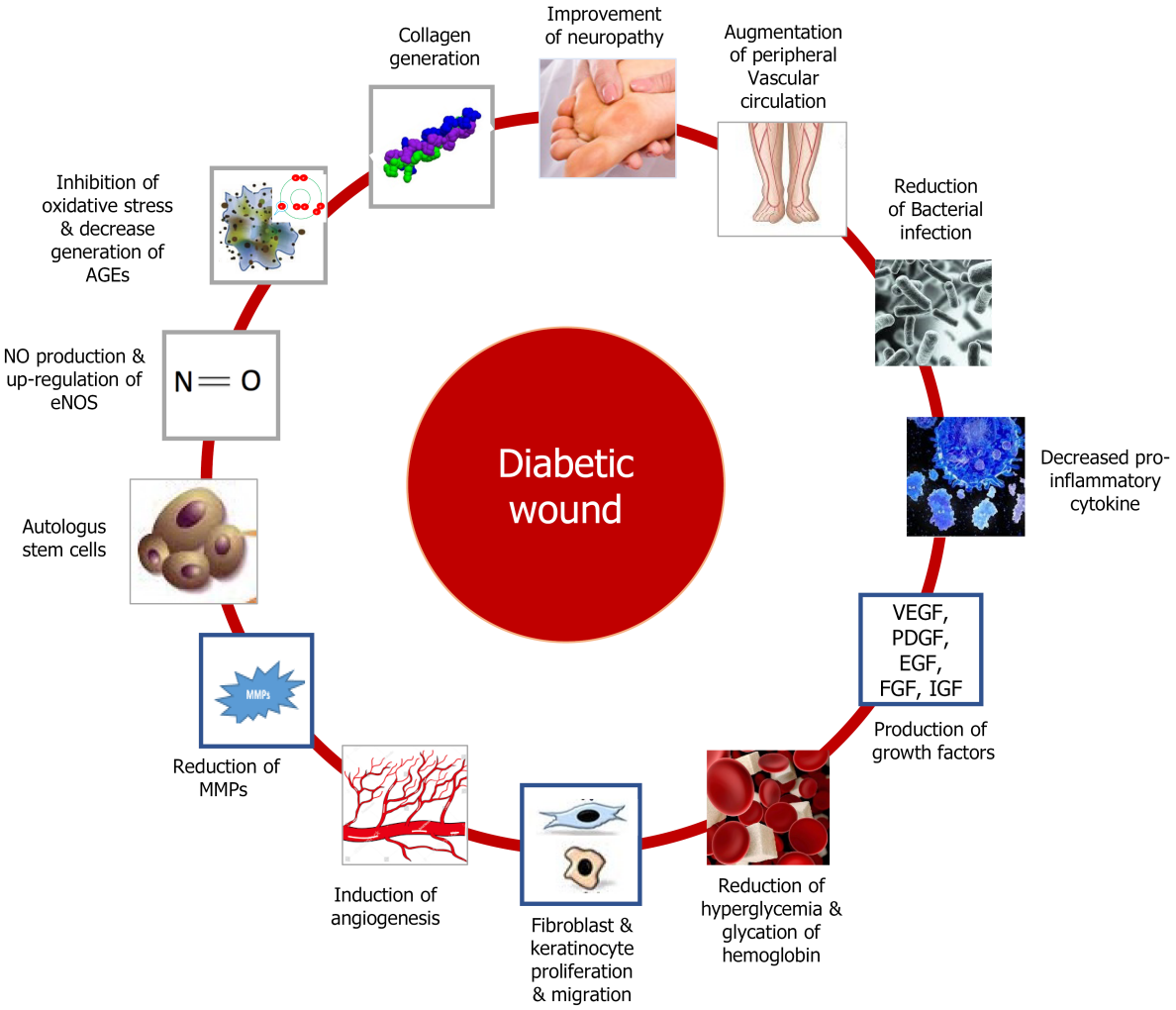
PRP is an important treatment approach investigated in DFU. A systematic review with meta-analyses of 10 studies reported that PRP promotes chronic diabetic wound healing (RR = 1.32; 95%CI: 1.11-1.57, I^2 = 15%) by reducing the volume and time of wound healing[160]. PRP may act *via* promoting the proliferation of wound cells, upregulation of cyclin A and cyclin-dependent kinase 4 proteins, modification of macrophage phenotype, reduction of TNF-α, enhancement of TGF-β and VEGF, increased secretion of fibroblast of collagen type I and III[161].

Table 1 Clinical trial of few therapeutic agents/approaches in the management of diabetic wound

Ref.	Type	Drug/ product /approach investigated	Type and number of participants	Type of wound	Observation
[132]	Homeopathic medicine	Silicea, Sulphur, Lycopodium, Arsenic album, Phosphorus	Observational study, 156 patients	DFU	Ulcer assessment score reduced significantly ($P < 0.05$) after treatment. Silicea, sulphur ($n = 11$), lycopodium, arsenic album, phosphorus were found more effective. Although, the effect of homeopathic therapy alone is difficult to establish
[133]		Silicea	Observational study, 22 patients	DFU	Positive and encouraging result of silicea in ulcer healing was reported. DFU assessment score was measured, and mean symptom scores at the end of the treatment were found to reduce significantly ($P < 0.05$)
[134]	Herbal Products	ON101 cream (contain extract of *Plectranthus amboinicus* and *Centella asiatica*)	Phase 3 RCT, 236 participants	DFU	Incidence of complete healing in ON101 and comparator group was 60.7% and 35.1% respectively. Although, the number of adverse events in the ON101 group was 7 *vs* 5 in the comparator group. ON101 produced a better healing effect compared to absorbent dressing alone
[135]		Intravenous Semelil (a naive herbal extract)	RCT, 25 participants	DFU	Mean foot ulcer surface area reduced significantly in semelil (i.v. route) group and the average wound closure in semelil group was significantly more than control group (64% *vs* 25%, $P = 0.015$). Semelil in combination with conventional therapy showed better effect than conventional therapy
[136]		Olive oil	Double blind RCT, 34 participants	DFU	Degree of ulcer, color, surrounding tissues, the status of ulcer and ulcer drainages were evaluated after topical application of olive oil. Complete ulcer healing in the treatment group was significantly better than the control group (73.3% *vs* 13.3%, $P = 0.003$). Olive oil treatment significantly reduced ulcer area and ulcer depth. Olive oil in combination with routine care was better than routine care alone
[137]		Polyherbal formulation (contain *G. glabra*, *M. paradisiaca*, *C. longa*, *P. odaratissimus*, *A.e vera*, *C. nucifera oil*)	Open label, phase III, comparative study, 40 participants	DFU	Polyherbal formulation was found effective similar to that of standard silver sulphadiazine cream
[138]		Semelil (ANGIPARS™, contain *Melilotus officinalis*)	Clinical study, 10 participants	DFU	ANGIPARS™ was found effective in reduction of wound size by at least 50% during 8 wk period
[139]	PRP	PRP gel	Single-arm clinical trial, 100 participants	DFU	PRP therapy (2 mL/cm^2 of ulcers) was found highly effective in the treatment and healing of non-healing chronic DFUs
[140]		PRP	Prospective RCT, 20 participants	DFU	Wound healing time was estimated as 8 wk which is superior to the control group. People treated with PRP it found more effective in wound healing
[141]	Human EGF (hEGF)	Recombinant hEGF	Prospective, open-label trial, crossover study, 89 participants	DFU	Wound healing was noted within an average of 46 d in patients who were treated with 0.005% EGF twice a day. Topical application of hEGF combined with hydrocolloid dressing showed promoting healing effect in chronic DFU
[142]		Regen-D 150 (hEGF gel-based product)	RCT, 50 participants	DFU	Complete ulcer healing was detected in 78% population against 52% population in the placebo group. Collagen and fibroblasts were significantly developed in the treated group. The application of hEGF can be helpful to promote wound healing and in preventing leg amputations
[143]	PDGF	rhPDGF	RCT, 50 participants	DFU	Wounds contracted more in rhPDGF-treated group compared to the control group (38.55% *vs* 12.79%; $P \leq 0.001$). Dressing with rhPDGF was found more effective and promoted safe wound healing
[144]		PDGF gel	RCT, 29 participants	Diabetic lower extremity ulcer	100% of ulcers were healed in subjects who received PDGF compared to 76.4% of wound healing in placebo group. The study confirms the effectiveness of PDGF gel
[145]	FGF	bFGF	Double-blind RCT, 150 participants	Non-ischaemic diabetic ulcer	Wound cure rate in 0.001% bFGF, 0.01% bFGF and control group was 57.4%, 66.7% and 46.8%. The area of the ulcer was also significantly decreased in bFGF treated groups. bFGF accelerates wound healing in diabetic people
[146]	Oxygen therapy	Topical oxygen therapy	RCT, 145 participants	DFU	A significant decrease in wound area was reported in the topical oxygen therapy + standard care group (70%) compare

					to the standard care group (40%) Addition of topical oxygen therapy with standard care facilitates wound closure in a better way
[147]		Hyperbaric oxygen therapy	RCT, 75 participants	Chronic DFU	Complete healing of ulcer index was reported in 52% of participants who received hyperbaric oxygen therapy after 1 year, which was 29% in the placebo. Adjunctive treatment with hyperbaric oxygen therapy may facilitate the healing of foot ulcers
[148]	NPWT	NPWT	Prospective randomized study, 55 participants	DFU	Granulation tissue formation (91.14% *vs* 52.61%, $P < 0.001$) and a decrease in the size of ulcer size (40.78% *vs* 21.18%, $P = 0.008$) were reported in the NPWT group after 14 d. Duration of hospital and time for complete coverage of the wound with granulation tissue was significantly less in the NPWT group. NPWT led to an early decrease in the size of the ulcer, formation of more granulation tissue, and wound healing
[149]		NPWT	RCT, 55 participants	DFU	The rate of ulcer healing was found higher in the NPWT group (*P*-value 0.01). NPWT leads to a higher rate of healing, and causes a significant decrease in ulcer surface area, size, and depth of the wound, reducing the risk of amputations
[150]	Phototherapy	LLLT	RCT, 23 participants	DFU	Ulcers size reduced significantly in 4th week in LLLT group ($P = 0.04$). More patients healed completely in LLLT group compared to the placebo group. Meantime of complete healing in patients treated with LLLT was 11 wk *vs* 14 wk in placebo patients. LLLT promotes the healing process of chronic DFU, and reduces the time required for wound healing
[151]		LLLT	RCT, 56 participants	DFU	Increment in total hemoglobin was more using the highest intensity configuration compare to the lower intensity setup in patients with DFU. A decrease in the very-low frequency/low frequency ratio, slightly more than the highest intensity in DFU people was observed. LLLT was found to increase blood flow and regulation of the autonomic nervous system in patients with DFU
[152]	ESWT	ESWT	Single-blinded RCT, 38 participants	DFU	Patients received shock wave therapy 2/week for a total of 8 treatments. Average healing time was lower in ESWT-group when compared with the control group (64.5 *vs* 81.17 d, $P < 0.05$). AT 20 wk, 54% of ulcer healed completely in ESWT-groups compared to 28.5% in the control group
[153]		ESWT	Prospective RCT, 23 participants	DFU	At 7 wk, the mean reduction in ulcer area was 34.5% (CI, 0.7-68.3) in the ESWT group and 5.6% (CI, -42.1-53.3) in the control group. ESWT also enhances tissue oxygenation
[154]	Stem cell therapy	Topical application of MSC	Clinical case study of three patients	Neuropathic DFU	MSCs at low doses enhance the re-epithelialization of DFU. MSCs may start early to reduce overall wound closure time
[155]		HUCMSCs	RCT, 56 participants	DFU	Patients in HUCMSCs (endovascular infusion and injection around the foot ulcer) experienced greater and betterment in skin temperature, transcutaneous oxygen tension, ankle-brachial pressure index, and claudication distance. Three months after treatment significant enhancement in neovessels, and complete or gradual ulcer healing was observed in the experimental group
[156]	NO generating approach	EDX110 (nitric oxide generating medical device)	RCT, 135 participants	DFU	At 12 wk, EDX110 use resulted in 88.6% reduction in median wound area compared to 46.9% for the control group ($P = 0.016$). EDX110 was found to improve foot wound healing in diabetic people significantly by reducing the ulcer area
[157]	Other Approaches	Bemiparin (low MW heparin)	RCT, 70 participants	DFU	In bemiparin group, the ulcer improvement rate was 70.3% compared to 45.5% in the placebo group. Though, complete healing rates found similar in both groups at 3 mo were, as were the number of adverse events. Bemiparin is better than a placebo in the management of DFU and has few side effects
[158]		Honey dressing	RCT, 348 participants	DFU	In 75.97% of cases wound healed completely after honey dressing in comparision to 57.39% of case in the saline dressing group. The homey dressing also reduced the median wound healing time (18.00 d) compare to the control group (29.00 d). Honey is an effective dressing substance compared to conventional dressings
[159]		Omega-3–rich fish skin grafts	RCT, 49 participants	DFU	At 12 wk, 67% of foot wounds were completely closed compared with 32% in the standard care group. Study findings indicated that fish skin graft is useful in the treatment of chronic DFUs that do not heal with standard treatment

DFU: diabetic foot ulcers; RCT: randomized controlled trials; PRP: platelet-rich plasma; hEGF: Human endothelial growth factor; rhPDGF: Recombinant human platelet-derived growth factor; PDGF: platelet-derived growth factor; bFGF: Basic fibroblast growth factor; NPWT: Negative pressure wound therapy; LLLT: Low-level laser therapy; ESWT: Extracorporeal shock wave therapy; MSC: Mesenchymal stromal cell; HUCMSCs: Human umbilical cord mesenchymal stem cells; NO: nitric oxide.



DOI: 10.4239/wjd.v13.i9.696

Figure 3 Plausible drug targets for diabetic wound. AGEs: Advanced glycation end-products; VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor.

A systematic review of 26 randomized controlled trials (RCTs) examined the usefulness of different recombinant proteins and growth factors in the treatment of DFU. EGF can be used as intralesional injection, topical cream, or gel. This meta-analysis reported that EGF significantly improves wound healing and the study failed to find a significant effect of EGF in reducing the risk of amputation[162]. Decreasing the level of circulating C-reactive protein, decreasing NFκB1, TNF-α, and IL-1a expression, and increasing the wound expression of platelet-derived growth factors (PDGF)-B, cyclin-dependent kinase 4, P21, TP53, angiopoietin 1, collagen 1A1, MMP-2, and TIMP-2 after the treatment with hEGF may be linked to its beneficial effect[161]. Recombinant human PDGF (rhPDGF) also exhibits their beneficial effect in several clinical studies[143,144], but few clinical studies also failed to find statistically significant effects of rhPDGF compared to the control group[163,164] when used to cure DFU. The effectiveness of PDGF may be related to the promotion of fibroblast and leukocyte migration, and synthesis of extracellular matrix[161]. FGF, a family of cell signaling proteins, is found to stimulate angiogenesis, induce fibroblasts proliferation, and promote wound healing. About 23 subtypes of FGF from 7 subfamilies were identified[165]. Some clinical studies have reported the beneficial effect of FGF in diabetic wound[145], some studies failed to report the advantageous effect of FGF over the placebo group[166], while clinical studies reported the beneficial effect of combined use of human EGF and acidic FGF on wound healing in the later stage[167]. Very few clinical studies evaluated the effectiveness of granulocyte-colony-stimulating factor, topical telbermin, epoetin-β, talactoferrin, TGF-β2 in

the diabetic wound. But the results are inconsistent[162].

Oxygen therapy is found to improve cell metabolism and energy, decrease proinflammatory cytokines and ROS, and promote the synthesis of matrix and wound repair[161]. Few clinical studies confirmed the beneficial effect of hyperbaric as well as topic oxygen therapy in promoting diabetic wound[146,147]. A systematic review and meta-analysis of 20 RCTs and 1263 trials analysed that hyperbaric oxygen therapy confer benefits in DFU treatment by increasing the healing of ulcers (relative risk, 1.901; 95%CI: 1.484-2.435, $P < 0.0001$), reducing healing time, and also decrease the risk of major amputation[148].

It was evaluated that NPWT results in macro- and micro-deformation that stimulate different wound healing cascade like promotion of tissue granulation, epithelialization, proliferation of vessel, neo-angiogenesis, pro-angiogenic condition, removal of surplus extracellular fluid, anti-inflammatory effect, increase expression of VEGF, FGF2, modification of circulating micro-RNAs, alteration of DNA methylation of genes linked with wound repair[161,168]. Liu *et al*[169] in their study (meta-analysis of 11 RCTs) concluded that NPWT is a safe, cost-effective and effective strategy in the treatment of DFU, while Liu also evaluated 11 RCTs and found that compared with wound dressings NPWT may enhance the proportion of wounds healed, decrease the time of postoperative foot wound healing[170].

LLLT was found to increase blood flow and regulation of the autonomic nervous system in patients suffering from DFU[151]. Reduce wound inflammation, enhance fibroblasts and angiogenesis[161], which may play an important role in diabetic wound healing. A metanalysis of 13 RCTs that included 413 patients concluded that LLLT significantly enhanced complete healing rate (RR = 2.10, 95%CI: 1.56-2.83, $P < 0.00001$), decreased wound ulcer area, and reduce mean healing time of wound healing in patients suffering from DFU[171].

Huang *et al*[172] performed a meta-analysis of 8 RCTs and concluded that ESWT treatment reduced wound surface area in greater proportion, enhances re-epithelialization and can reduce treatment inefficiency. ESWT is useful as an adjuvant strategy in the management of DFUs, which can improve the complete wound cure rate and reduce the healing period of DFUs. ESWT may enhance the angiogenesis process, decrease macrophage number, and enhance the production of macrophage of growth factors from macrophages that help in the healing of wound[161].

In light of current evidence, it can be suggested that stem cell-based therapy (delivery through both local and systemic route is effective to heal DFU and considered a promising regenerative medicine, and mechanisms of stem cell therapy include improved angiogenesis, decrease inflammation, ameliorating neuroischemia, improved collagen deposition, *etc.*[173].

Wound healing in diabetic people can be promoted by providing endogenous or exogenous NO. Products (*i.e.*, patches/ matrices) that release NO are used to treat diabetic wounds by different mechanisms like enhancement of angiogenic activity, endothelial cell proliferation, conferring antimicrobial substances, and promoting cell migration to the injured site[174]. Only a few clinical studies have reported the beneficial effects of NO-releasing devices, and several products are in the clinical trial.

Homeopathic medicines (like silicea, sulphur, lycopodium, and arsenic) were also investigated in the clinical trial and concluded that homeopathic medicine may be useful in the management of diabetic wounds[132,133]. Several other products, like honey, fish skin grafts, *etc.*, were also clinically investigated for their beneficial effects against DFU[158,159]. Further, several herbal products were also successfully investigated in the treatment of diabetic wound[134,135,136-138].

MEDICINAL PLANTS AND PHYTOCONSTITUENTS IN DIABETIC WOUND

Medicinal plants and phytoconstituents always represent an important, effective and alternative treatment strategy to cure diseases. Phytochemicals have showed their effectiveness in different diabetes complications. Anti-inflammatory mechanism of phytochemicals is considered important in the management of diabetes wound. Epigallocatechin gallate in pre-clinical investigation was found to reduce reduced levels of IL-1β, TNF-α and IL-6, producing inhibition of Notch signaling and accumulation of macrophage at a wound site. Kaempferol, is an important dietary flavonoid found to exert different pharmacological activities, including antioxidant, anti-inflammatory and cardioprotective activity. An ointment containing Kaempferol was found effective in diabetic excisional and non-diabetic incisional wounds in experimental animals[175]. Flavonoids an important class of phytoconstituents, exerted anti-inflammatory and antioxidant effect, and also enhances angiogenesis and re-epithelialization. Preclinical trials found the effectiveness of isoliquiritin, isoflavonoid, naringenin, dihydromyricetin, dihydroquercetin, quercetin, hesperidin, kaempferol, proanthocyanidins, icariin, puerarin, rutin, genistein, luteolin, rutoside, silymarin, daidzein, genistein, and epigallocatechin gallate to cure wound[174]. Flavonoids positively regulate MMP-2, MMP-8, MMP-9, MMP-13, Ras/Raf/MEK/ERK, PI3K/Akt, and NO pathways. Phytochemicals are found to reduce oxidative stress, expression/release of proinflammatory/inflammatory cytokines, *i.e.*, TNF-α, IL-1β, IL-6, NF-κB and upregulate IL-10 and antioxidant enzymes. Flavonoids also act on macrophages, fibroblasts and endothelial cells by facilitating expression/release of TGF-β1, VEGF, angiopoietin, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains, and small mothers against

decapentaplegic 2 and 3[176]. Oguntibeju[177] in his paper highlighted different medicinal plants like *Rosmarinus officinalis*, *Carica papaya*, *Radix rehmanniae*, *Annona squamosa*, *Catharanthus roseus*, *Centella asiatica*, *Acalypha langiana*, *Hylocereus undatus*, *Punica granatum*, *Aloe vera*, and *Martynia annua* that has been investigated in the treatment of diabetic wound. Benefits of the plants may link to different mechanisms like an increase in fibroblast cell, fibroplasia, increase in collagen formation, enhancement of tissue regeneration, angiogenesis, antimicrobial, anti-inflammatory and antioxidant effect. A recent clinical study established the effectiveness and safety of nano-hydrogel embedded with quercetin and oleic acid when used in the management of lower limb skin wound in diabetic patients. The formulation effectively treated the wound and reduce the wound healing time compared to the control group[178]. Infections caused by different microbes like *Staphylococcus aureus*, *Streptococcus* β-hemolytic, *Pseudomonas aeruginosa*, *Peptostreptococcus spp.*, *Proteus spp.*, *Prevotella spp.*, *Bacteroides spp.*, *Clostridium spp.* and anaerobes are posing a serious situation in diabetic people[126]. Medicinal plants and phytochemicals with antimicrobial activity may also play an important role in the management of diabetic wound. Several plants have shown their potential against the microbial strain responsible for infection in the diabetic wound[126]. Formulation designed with *Momordica charantia*, *Actinidia deliciosa*, *Aloe vera*, *citrus fruits*, *Sida cordifolia*, *Nigella sativa*, *Curcuma longa*, and *Azadirachta indica* has shown their potential in the treatment of diabetic wound[126]. Isoflavones isolated from plant sources were also found to be effective against DFU bacteria[179] Phytofabricated silver nanoparticles (*Aerva lanata* reduced silver nanoparticles) at 20 μg/mL were found highly effective against multi antibiotic-resistant DFU isolates like *E. coli*, *P. aeruginosa*, *S. aureus, S. subtilis*. Identified phytochemicals of *A. lanata* include rutin, quercetin, kaempferol, gallic acid and ellagic acid[180]. The use of phytoextracts/active compounds may be considered as an important strategy for addressing the wound problem associated with DM in a better way.

CONCLUSION

Diabetes adversely acts on the phases of normal wound healing phases, i.e., hemostasis, inflammatory phase, proliferative phase, re-epithelialization and remodeling phase, and poses a big burden on the quality of life of a diabetic individual. Hyperglycemia can trigger oxidative stress, increase inflammatory cytokines, interrupt angiogenesis, decrease the functioning of fibroblast and keratinocyte, induce neuropathy associated events, increase MMPs, and reduce TIMPs that is responsible for impaired states of wound healing. An understanding sequence of the molecular and cellular cascade, epigenetic mechanisms, microbial perspective, complexity and plasticity of impaired wound healing in diabetic conditions is required for targeted research focusing on treatment of diabetic wound. Pharmacotherapy/strategy involving angiogenesis stimulation, growth factors, cytokines modulators, MMP inhibitors, ECM stimulators, anti-inflammatory drugs, antidiabetic agents, antimicrobial drugs, debridement, offloading, PRP, oxygen therapy, NPWT, LLLT, ESWT, stem cells, bio-engineered substitutes, and various natural-based products have shown their benefit. There has been a lack of quality-based evidence of efficacy of different adjuvant therapies tested through different clinical trials, thus more structured and quality studies are required. Indeed, the utilisation of medicinal plants/products in diabetic wound care holds prodigious potential in the future, and the development of innovative pharmaceutical formulations for advanced wound care is equally critical. Effective diabetic wound management necessitates a combination of techniques, including medication and non-pharmacological intervention. Hence, the treatment strategy of the future can only succeed if research concentrated on plausible drug targets after comprehending the inherent pathological complexities, evaluating non-pharmacological approaches through well-designed clinical trials, and targeting natural sources for new drug development.

FOOTNOTES

Author contributions: Chakraborty R, Borah P, Sen S, Dutta PP performed data accusation and writing; Borah P, Dutta PP prepared the figures; Chakraborty R provided the input in writing the paper; Sen S designed the outline and coordinated the writing of the paper.

Conflict-of-interest statement: There is no conflict of interest associated with any of the authors contributed their efforts in this manuscript.

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 717-728

DOI: 10.4239/wjd.v13.i9.717

ISSN 1948-9358 (online)

MINIREVIEWS

Potential role of *Limosilactobacillus fermentum* as a probiotic with anti-diabetic properties: A review

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Specialty type: Microbiology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bala C, Romania; Shi J

Received: March 2, 2022
Peer-review started: March 2, 2022
First decision: April 17, 2022
Revised: April 29, 2022
Accepted: August 14, 2022
Article in press: August 14, 2022
Published online: September 15, 2022

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Abstract

Oxidative stress, inflammation, and gut microbiota impairments have been implicated in the development and maintenance of diabetes mellitus. Strategies capable of recovering the community of commensal gut microbiota and controlling diabetes mellitus have increased in recent years. Some lactobacilli strains have an antioxidant and anti-inflammatory system capable of protecting against oxidative stress, inflammation, and diabetes mellitus. Experimental studies and some clinical trials have demonstrated that *Limosilactobacillus fermentum* strains can beneficially modulate the host antioxidant and anti-inflammatory system, resulting in the amelioration of glucose homeostasis in diabetic conditions. This review presents and discusses the currently available studies on the identification of *Limosilactobacillus fermentum* strains with anti-diabetic properties, their sources, range of dosage, and the intervention time in experiments with animals and clinical trials. This review strives to serve as a relevant and well-cataloged reference of *Limosilactobacillus fermentum* strains capable of inducing anti-diabetic effects and promoting health benefits.

Key Words: Diabetes Mellitus; Gut dysbiosis; Oxidative stress; Probiotics; *Limosilactobacillus fermentum*

Core Tip: This review strives to serve as a relevant and well-cataloged reference of L. fermentum strains with aptitudes of inducing anti-diabetic effects and health-promoting benefits to the host envisaging their wide applicability to diabetes control.

Citation: Lacerda DC, Trindade da Costa PC, Pontes PB, Carneiro dos Santos LA, Cruz Neto JPR, Silva Luis CC, de Sousa Brito VP, de Brito Alves JL. Potential role of *Limosilactobacillus fermentum* as a probiotic with anti-diabetic properties: A review. *World J Diabetes* 2022; 13(9): 717-728
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/717.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.717

INTRODUCTION

Diabetes mellitus (DM) is a chronic non-communicable disease that affects millions of people and has become one of the leading causes of death worldwide[1,2]. The Diabetes Atlas published by the International Diabetes Federation estimated that 537 million adults worldwide had type DM in 2021[3]. Associated to this prevalence, clinical management of DM has elevated the costs of the health system, increasing by 316% in the last 15 years[3,4]. One of the etiological factors of this metabolic disorder includes long-term inappropriate diet such as regular consumption of sugary drinks, red meat, and low consumption of whole grains and fiber. In addition, smoking, physical inactivity, history of gestational diabetes or delivery of newborns > 4 kg weight, medications such as statins, thiazides, and beta-blockers, psychosocial stress, and depression have been described as risk factors for DM[5-7].

Clinical features and laboratory findings of DM include changes in body weight, increased blood glucose, insulin resistance, development of lipid metabolism disorder, polyuria, polydipsia, visual disturbances, ketoacidosis, and hyperosmolar non-ketoacidotic syndrome with risk of coma[8,9]. When uncontrolled, diabetes can induce grave complications, including death[10]. Insulin resistance in sensitive tissues such as liver, muscle, and adipose tissue and β-cell dysfunctions are the main factors involved in initiating and progressing the pathophysiology of type 2 DM[5,11]. Moreover, it has been reported that gut microbiota (GM) impairment plays a crucial role in developing DM[12].

DM patients show an altered intestinal microbiota resulting from an increase in opportunistic bacteria and Gram-negative toxin-producing bacteria that alter metabolism energetic[13]. Furthermore, the accumulation of gut-derived pro-inflammatory molecules, including lipopolysaccharide (LPS), peptidoglycans, and flagellin, appear to accelerate the inflammatory response in patients with DM[14]. Deregulation of the GM, also called dysbiosis, promotes intestinal permeability and energy homeostasis changes, causing metabolic endotoxemia, inflammation, hyperglycemia, and hyperlipidemia[15,16]. Dysbiosis impairs the integrity of the intestinal wall and allows the translocation of toxins from the intestinal lumen into the systemic circulation, promoting inflammation, autoimmunity, and oxidative stress that can lead to β-cell destruction or insulin resistance[17,18].

The findings involving the association between gut dysbiosis and DM reinforce the importance of gut-targeting approaches in the treatment of DM[19-21] Strategies capable of recovering the community of commensal GM and controlling DM have recently increased. Probiotic therapy has begun to be used to improve GM composition and management of DM[22,23]. Given this scenario, the identification of new potentially-probiotic strains with anti-diabetic properties is essential for the development of new probiotic products and testing in well-controlled trials.

Among *Lactobacillus* species, strains of *Limosilactobacillus fermentum* (*L. fermentum*) has been reported to exert probiotic properties due to its ability to improve GM composition, reduce blood cholesterol, modulate the intestinal immune system, stimulate the release of immunoglobulin A, reduce intestinal inflammation, and increase the activity of antioxidant enzymes[24-27]. Although early studies have identified anti-diabetic properties in some *L. fermentum* strains, an in-depth review focusing on *L. fermentum* strains as a potential anti-diabetic has not been found in the available literature to the time of this writing[28,29].

This present literature review focuses on the emerging findings of experimental and clinical studies that have used *L. fermentum* supplementation to prevent or treat complications of DM. To investigate the effectiveness of *L. fermentum* more thoroughly, we focus on the type of strain, source of probiotics, dosage, duration of treatment, and the primary outcomes reported.

PROBIOTIC THERAPY IN THE TREATMENT OF METABOLIC DISORDERS

Probiotics are live microorganisms that confer host health benefits when administrated adequately. Probiotics have significant importance in the industrial economy and are among the most consumed food supplements worldwide[30]. Experimental studies and clinical trials have documented that probiotics can modulate the GM, inducing beneficial effects and increasing overall wellness[28,31,32]. Over the last few years, studies on probiotics have been growing sharply due to their beneficial health effects, which have been used as adjuvant therapy for metabolic disorders[33]. A number of preclinical and clinical studies have investigated the effectiveness of probiotics by evaluating the intestinal microbiota after probiotics use, showing promising results in treating metabolic diseases[31].

Impairment in commensal homeostasis of GM and intestinal functional capacity, called gut dysbiosis, is associated with the development of metabolic diseases such as colitis, obesity, liver, obesity, and DM [34]. Thus, a probiotic may be able to relieve GM dysbiosis, through various mechanisms including improvement in the composition and diversity of the GM, induction of immunomodulation, protection against physiological stress, and pathogen suppression[35]. Probiotics also promote health benefits to the host through other mechanisms of action, such as the production of organic acids, including lactic acid and short-chain fatty acids (SCFA) (mainly acetate, propionate, and butyrate)[36,37]. Another mechanism reported is the capacity of probiotics to protect the integrity of the intestinal wall by stimulating mucin production and upregulating tight-junction claudin, occludin, and zonulin protein expression[37]. Furthermore, probiotics are also responsible for producing small molecules with systemic effects essential for maintaining vital functions, such as cortisol, serotonin, gamma-aminobutyric acid (GABA), tryptophan, histamine derivatives, satiety hormones, and conjugated linoleic acid[37].

Coupled with the mechanisms mentioned above, some experimental and clinical evidence has demonstrated that probiotics have anti-inflammatory and antioxidant properties[27,37,38]. This antioxidant capacity results from signaling pathways that produce antioxidant enzymes and molecules, reducing serum and tissues levels of oxidative stress[38,39]. Concerning their anti-inflammatory properties, probiotics have been reported to reduce inflammatory markers, including LPS, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, as well as to promote an increase of anti-inflammatory markers, such as IL- 10.

LIMOSILACTOBACILLUS FERMENTUM, LEAKY GUT AND DIABETES MELLITUS

Probiotics have shown satisfactory results as an adjunct treatment in DM[40,42]. Both single strain, combined with other foods, and multiple strain probiotics can be used as supplements. Among more effective probiotic strains, the therapeutic potential of *L. fermentum* has been investigated for adjuvant management of DM[40,48,55].

L. fermentum is a Gram-positive, rod- or coccoid-shaped, heterofermentative, and anaerobic or aerotolerant bacteria found in fermented cereals and other fermenting plant materials, dairy products, manure sewage and feces, and the human vagina[41]. The *Lactobacillus* genus is widely used as an intestinal modulator due to its safety and probiotic activity[40]. Among these bacterial groups, *L. fermentum* is a well-studied species, mainly due to its action in improving metabolic function and oxidative stress, which may be considered for DM management[26,27,42].

DM is one of the main metabolic diseases related to leaky gut, oxidative stress, and chronic inflammation. GM impairment has been described in the pathogenesis of DM and metabolic syndrome. Due to the high mortality rate of patients with of DM and this direct relationship with intestinal health, the number of studies involving probiotic therapy has increased in recent years. *L. fermentum* has been proven to alleviate metabolic disorder-related symptoms, including improvement in glucose and insulin levels, control of the lipid profile, to decrease in pro-inflammatory cytokines and to increase antioxidant capacity[27,41,43]. However, these protective responses need to be further investigated in clinical studies to elucidate the responsiveness of *L. fermentum* therapy in DM patients.

Most diabetes treatments, particularly drug therapies, use agents that act directly on signaling pathways to regulate glucose. Because of this, it is pertinent to explore therapies that adjunctively attenuate deregulation of GM, such as probiotics[44]. Among the main harmful effects in GM induced by DM, gram-negative bacteria in the colon increase the concentration of LPS in the lumen. LPS causes high production of free radicals, increasing intestinal permeability and generating a systemic chronic inflammatory process. This pro-inflammatory state is a critical mechanism in the genesis of chronic diseases, such as DM[38,40]. Additionally, GM imbalances observed in DM patients are characterized by changes in the composition of SCFAs, including increasing acetate levels and decreasing butyrate production. As a consequence, there may be acetate excess and reduction of butyrate, caused by dysbiosis, and impaired blood glucose homeostasis[45].

On the other hand, *L. fermentum* manipulation could attenuate GM imbalance, which may decrease DM complications. Considering the inversely proportional relationship between butyrate and acetate levels and the effects of excess acetate on the worsening of DM, keeping these fatty acids in balance

becomes an important way to assist glycemic control[45]. Increased butyrate production by *L. fermentum* regulates acetate production, preventing increased hepatic gluconeogenesis and insulin resistance. Additionally, the increase in butyrate production resulting from *L. fermentum* supplementation may repair enterocyte tight junctions and improve intestinal permeability[46]. Experimental evidence has revealed that increasing levels of SCFA, especially acetate and succinate, decreases cellular damage of enterocytes, leading to a reduction in inflammation state, oxidative stress, and leaky gut in DM-induced rodents[28].

Another antidiabetic property of *L. fermentum* is to maintain normal levels of the intestinal hormone GLP-1[47]. GLP-1 has been shown to stimulate proliferation and prevent apoptosis of pancreatic beta cells, upregulating insulin synthesis and promoting a reasonable glycemic control[29,36]. In the liver, GLP1 decreases gluconeogenesis and stimulates glycolysis, contributing to reducing glycemic levels in individuals with DM. The main consequence of reducing these peptides is the exacerbation of hunger, the search for palatable food, and the preference for hypercaloric foods, which can be a predisposing factor for developing obesity and insulin resistance[30]. Leaky gut also generates chronic low-grade inflammation in organs such as the liver, skeletal muscle, and adipose tissue, causing metabolic changes such as hyperglycemia and dyslipidemias. *L. fermentum* also promotes benefits in these organs because it stimulates the synthesis of the fasting-induced adipose factor, a protein that regulates the function of the LPL enzyme and prevents hepatic steatosis and dyslipidemia, common in diabetic subjects[48]. Therefore, it is suggested that *L. fermentum* may improve intestinal permeability, normalize GLP-1 Levels, and reduce DM complications.

Another important action of *L. fermentum* is to reduce oxidative stress and glycation. Studies indicate that pathophysiological findings of DM, including macular degeneration, vascular endothelial injury, hepatic fibrosis, renal failure, are related to the glycation process. This process occurs when circulating glucose binds to proteins, inactivating them and increasing inflammatory cytokines such as interferon-gamma (IFN-γ), IL-6, and IL-4. The main biochemical marker for glycation is glycated hemoglobin (HB1ac), but this process can occur with any protein, including antioxidant enzymes. When glycation events occur more expressively, oxidative stress is even higher due to the increase in reactive oxygen species (ROS) and inactivation of the enzymatic antioxidant systems, such as superoxide dismutase (SOD) and glutathione peroxidase[40,49].

Conversely, the administration of *L. fermentum* decreased the glycation events and oxidative stress through the increasing production of ferulic acid (FA). This potent antioxidant metabolite can significantly reduce ROS formation and prevent glycation events. This mechanism is related to decreasing inflammatory markers, Hb1ac, and serum glucose. High levels of FA are also related to lower cardiometabolic risk in diabetic individuals[40,44].

To evaluate the effectiveness of *L. fermentum,* the following sections refer to the findings on the antidiabetic properties of different strains of *L. fermentum,* investigated in preclinical and clinical studies.

ANTI-DIABETIC PROPERTIES OF DIFFERENT STRAINS OF *LIMOSILACTOBACILLUS FERMENTUM*

We investigated studies that analyzed the role of *L. fermentum* administered singly or combined with other therapies to alleviate DM complications. Among ten of the studies included, nine evaluated anti-diabetic properties in experimental studies using rats or mice. Only one clinical study assessed the anti-diabetic potential of probiotic intervention in women with gestational DM. Since the majority of beneficial effects following administration of *L. fermentum* come from animal studies, this present review investigated emerging findings of their potential role in DM management. The characteristics of the studies and the primary outcomes are summarized in Table 1 and Table 2, respectively.

L. fermentum LLB3

An experimental study revealed that treatment with *L. fermentum* LLB3 isolated from the bamboo shoot pickle and offered in fermented bitter melon (*Momordica charantia*), in a concentration of 1×10^7 CFU during 4 wk, reduced fasting glucose and postprandial blood glucose levels and increased SOD enzyme activity in rats subjected to type 2 DM induced by streptozotocin (STZ)[50]. This suggests that *L. fermentum* LLB3 might be considered an adjuvant therapy to attenuate type 2 DM-related symptoms[50].

L. fermentum HP3

Administration of a fermented *Hericium erinaceus* juice containing 10^9 CFU/mL of *L. fermentum* HP3 for 12 wk reduced weight gain, increased insulin level, and reduced hyperglycemia in diabetic mice induced by STZ[51]. In addition, treated mice showed lower levels of inflammatory cytokines, including IL-6, IL-17, and IFN-γ[51], suggesting that fermented *Hericium erinaceus* juice can be used as nutritional manipulation in the treatment of type 2 DM.

Table 1 Characteristics of the studies testing the anti-diabetic effect of *Limosilactobacillus fermentum* strains

Ref.	Type of study	Experimental groups	Source of probiotics	Dosage of probiotic	Duration of treatment
Hartajanie *et al*[50], 2020	Experimental: 24 male Sprague-Dawley rats at 8 wk and weighing 170-200 g	Diabetic group; Diabetic group + acarbose; Diabetic group + bitter melon; Diabetic group + fermented bitter melon	*L. fermentum* LLB3 was isolated from the bamboo shoot pickle	1×10^7 CFU *L. fermentum* LLB3	4 wk
Hu *et al* [35], 2019	Experimental: 4-wk-old male Kunming mice (18 ± 2 g) were used	Normal control group; Diabetic group; Positive drug control group; Diabetic group + fructose 1 6-bisphosphatase (low dose); Diabetic group + 1-Deoxynojirimycin (middle dose); Diabetic group + 1-Deoxyn-ojirimycin (high dose)	All probiotics were purchased from the Guangdong culture collection center	5×10^4 CFU/mL of each activated strain (*L. plantarum* + *L. fermentum*, *L. plantarum* + *L. mesenteroides*, *L. plantarum* + *S. cerevisiae*, *L. fermentum* + *L. mesenteroides*, *L. fermentum* + *S. cerevisiae*, and *L. mesenteroides* + *S. cerevisiae*)	4 wk
Chaiyasut *et al*[51], 2018	Experimental: male *Wistar* rats	Control group; Control group + *L. fermentum*; Control group + fermented *H. erinaceus* juice; Diabetic group; Diabetic group pretreatment and posttreatment treated with fermented *H. erinaceus* juice, *L. fermentum*, and insulin	*L. fermentum* HP3 was isolated from fermented Thai foods	*L. fermentum* HP3 in a concentration of 10^9 CFU/mL. *L. fermentum* HP3 was used with *H. Erinaceus* Juice	12 wk
Guilbaud *et al*[52], 2020	Experimental: 30 mice with 6 wk of age	Wild-type group;Wild-type group + *L. fermentum*;Diabetic group;Diabetic group + *L. fermentum*	Isolated from a fecal sample of one-year-old healthyE-stonian child	*L. fermentum* ME-3 in a concentration of 10^{10} CFU per 400 μL H_2O	12 wk
Archer *et al* [48], 2021	Experimental: 40 female *Wistar* rats	Control group; Diabetic group + high-fat diet; Diabetic group + high-fat diet + *L. fermentum*. MCC2759; Diabetic group + high-fat diet + *L. fermentum*. MCC2760	Isolated from fecal (*L. fermentum*. MCC2759) and from curd (*L. fermentum*. MCC2760)	Both isolated probiotics were offered in a concentration of 1×10^9 CFU/mL	4 wk
Ai *et al*[31], 2021	Experimental: 160 Male C57BL/6J mice with 6 wk of age	Control group; Diabetic group + high-fat diet; Diabetic group + defatted rice bran unfermented extracts; Diabetic group + pioglitazone; Diabetic group + high-dose of defatted rice bran fermentation extracts; Diabetic group + low-dose of defatted rice bran fermentation extracts	Isolated from Chinese rice noodle wastewater	The study evaluated the role of *L. fermentum* MF423. Dose of 100 μg/mL of defatted rice bran unfermented extracts	8 wk
Yadav *et al* [54], 2018	Experimental: 70 male *Wistar* rats with 8 ws old	Normal control group; Diabetic control group; Diabetic + normal diet supplemented with milk; Diabetic + *L. rhamnosus* MTCC5957; Diabetic + *L. rhamnosus* MTCC5897; Diabetic + *L. fermentum* MTCC 5898; Diabetic + *L.rhamnosus* 5957 and 5958 and *L. fermentum* MTCC 5898	The probiotics *L. rhamnosus* MTCC: 5957 and *L. rhamnosus* MTCC: 5897 were isolated from household curds. The probiotic *L. fermentum* MTCC: 5898 was isolated from the feces of breastfed human infants	All probiotic strains were offered in a dosage of 1×10^9 CFU	6 wk
Yousaf *et al* [55], 2016	Experimental: female mice of 6-8 wk, with an initial body weight of 21-23 g	Normal healthy mice; Diabetic mice; Diabetic mice + *Momordica charanti*; Diabetic mice + *Eugenia Jambolana*; Diabetic mice + *L. Fermentum*; Diabetic mice + *L. Fermentum* + Momordicacharanti + *Eugenia Jambolana*; Diabetic mice + Glucophage	*L. fermentum* fruit extracts of *Eugenia Jambolana* and *Momordica charantia* were isolated from local yogurt samples (Lahore, Pakistan)	*Momordica charantia* 200 mg/kg, and *Eugenia Jambolana* 100 mg/kg. The authors did not inform the concentration of *L. fermentum* (Gene Bank Accession KJ754019)	3 wk
Balakumar *et at*[49], 2018	Experimental: adult male C57BL/6J mice (age 8-10 wk)	Normal pellet diet; High-fat diet; High-fat diet + *L. rhamnosus*; High-fat diet + *L. plantarum* MTCC5690; High-fat diet + *L. fermentum* MTCC5689; High-fat diet + metformim; High-fat diet + vildagliptin	Isolated from Indian gut (Karnal, India)	*Lactobacillus* MTCC 5690 and MTCC 5689 in a concentration of 1.5×10^9 colonies/mouse/d	24 wk
Babadi *et al* [30], 2018	Clinical: primigravid women aged between 18 and 40 years, between the 24th and 28th week of gestation, diagnosed with gestational diabetes	Placebo group; Probiotic group	Probiotic supplements were produced by LactoCare®, Zisttakhmir Company (Tehran, Iran)	Probiotic capsule containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* and *L. fermentum* in a dosage of 2×10^9 CFU/g	6 wk

	mellitus

L. fermentum: *Lactobacillus fermentum*; *L. rhamnosus*: *Lactobacillus rhamnosus*; *L. plantarum*: *Lactobacillus plantarum*; *S. cerevisiae*: *Saccharomyce cerevisae*; *L. mesenteroides*: *Leuconostoc mesenteroides*; *H. erinaceus*: *Hericium erinaceus*.

Table 2 Primary outcomes of the studies testing the anti-diabetic effect of *Limosilactobacillus fermentum* strains

Ref.	Primary end-points
Hartajanie *et al*[50], 2020	↓ The fasting blood glucose; ↓ Postprandial blood glucose; ↑ In SOD concentrations
Hu *et al*[35], 2019	↓ Blood glucose levels; ↓ Insulin levels; Reversed insulin resistance; Improved serum lipid levels; Relieved gut dysbiosis
Chaiyasut *et al*[51], 2018	↓ Weight Gain; Improved insulin levels (↑ insulin); Recovery progress of hyperglycemia; ↓ HbA1c level (only with cointerventions); ↓ Inflammatory cytokines level
Guilbaud *et al*[52], 2020	↓ Weight Gain; ↓ Glycemic response 60-120 min; ↑ In HbA1c; ↓Weight of liver; ↓ FL-furosine levels in kidney ↓The expression of *TNF-α;* ↓The TG concentrations in liver; ↓ HDL and Non-HDL; Lower lipid droplets in liver.
Archer *et al*[48], 2021	↓ Blood glucose levels; Improved insulin levels (↑ insulin); ↓ levels of cholesterol, triglycerides, and LDL-C; ↓ The expression levels of *TNF-α*, and ↑ expression of *IL-10*; ↓ Expression of the *TLR4* receptor, ↑ Expression of tight junction protein *ZO-1*, endocannabinoid receptor *CB2* and GLP1, and ↑ Expression of *GLUT4* in MAT and muscle tissue; Showed accumulation of neutrophils around the portal tracts in liver tissue, and reduction in the glomerular injury in kidney sections
Ai *et al*[31], 2021	Inhibit the degree of weight loss; ↓ The fasting blood glucose; ↓ Blood glucose levels; ↓ Levels of total cholesterol and LDL and ↑ HDL levels; Ameliorate the damage to liver cells and significantly reduced the accumulation of lipid droplets; Upregulated the levels of SOD, T-AOC and GSH-PX, and reversed elevation of MDA; ↓ Damage in composition of gut microbiota[1]
Yadav *et al*[54], 2018	Inhibit the degree of weight loss; ↓ The fasting blood glucose; ↓ Consumption of food and liquids; ↑ In oral glucose tolerance; ↑ In liver weight; Improved insulin levels (↑ Insulin); ↓ HbA1c level; ↑ CAT, SOD activity in kidney and liver; ↓ Serum levels of total cholesterol, LDL-C, VLDL-C and triglycerides; ↓ The serum inflammatory index, cytokine levels (IL-6 and TNF-α); ↓ In the expression of the genes *G6Pase* and *pepck* in the liver
Yousaf *et al*[55], 2016	↑ Body weight; ↓ Blood glucose levels; Lipid profile: no effect on cholesterol, ↓ tryglyceride, LDL, slight increase in the level of HDL
Balakumar *et al*[49], 2018	↓ Body weight; ↓ Blood glucose levels; ↑ In oral glucose tolerance; ↓ HbA1c level; Improved insulin levels (↓ Insulin); ↑ levels of GLP-1; ↓ Cholesterol, triglyceride and LDL levels; ↑ HDL level; ↓ Plasma DX-4000–FITC; ↑ mRNA expression of epithelial tight junction *occludin* and *ZO-1*; ↓ Serum levels of LPS; ↓ Proinflammatory gene expression profiles *(IL6* and *TNFα*), ↑ *adiponectin* gene expression; ↓ Gene expression profiles of endoplasmic reticulum stress
Babadi *et al*[30], 2018	Downregulated gene expression of *TNF-α*; ↓ The fasting blood glucose; ↓ Serum insulin level; ↓ Insulin resistance; ↑ Insulin sensitivity; ↓ Levels of triglycerides, VLDL-cholesterol and total / HDL-cholesterol ratio, and ↑ levels of HDL-cholesterol; ↓ In plasma MDA; ↑ In plasma NO and total antioxidant capacity

[1]These results were obtained by rice bran fermented with Lactobacillus fermentum MF423.

SOD: Superoxide dismutase; HbA1c: Glycayed hemoglobin A; TNF-α: Tumor necrosis factor-alpha; TG: Triglyceride; HDL- C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; VLDL-C: Very-low-density lipoprotein cholesterol; IL-6: Interleukin-6; IL-10: Interleukin-10; TLR4: Toll-like receptor 4; ZO-1: Zonula occludens-1; CB2: Cannabinoid receptor type 2; GLP1: Glucagon-like peptide-1; GLUT4: Glucose transporter type 4; MAT: Mesenteric adipose tissue; T-AOC: Total antioxidant capacity; GSH-PX: Glutathione peroxidase; MDA: Malondialdehyde; CAT: Catalase; G6Pase: Glucose 6-phosphatase; Pepck: Phosphoenolpyruvate carboxykinase; FITC: Fluorescein isothiocyanate-dextran; LPS: Lipopolysaccharide; NO: Nitric oxide.

L. fermentum ME-3

A previous preclinical study investigated the anti-diabetic effect of *L. fermentum* ME-3 in genetically diabetic mice[52]. *L. fermentum* ME-3 was administered in mice at 6 wk of age, in a concentration of 10^{10} CFU, for 12 wk[52]. The treatment with *L. fermentum* ME-3 reduced body weight, inhibited expression of TNF-α, but did not improve glycemic control[52]. In addition, supplementation with *L. fermentum* ME-3 reduced the formation of glycation products, including FL-furosine levels in the kidney. However, the researchers found an increase in HbA1c, another marker of early glycation. The authors noted that while HbA1c reflects early glycation mainly in red blood cells, FL-furosine provides information on the extent of early glycation in fluids, tissues, and organs and offers a broader view of the early glycation status of the whole organism[52]. In summary, *L. fermentum* ME-3 has the therapeutic potential to reduce the formation of some glycation products in kidneys and attenuate some typical type 2 DM-related symptoms.

L. fermentum MCC2759 and MCC2760

Recently, an Indian research group analyzed the activity of the probiotic *L. fermentum* MCC2759 and MCC2760 on intestinal markers of inflammation using a high-fat diet model associated with the STZ-

induced diabetic model[48]. Both *L. fermentum* strains were administered in a concentration of 1 × 10^9 CFU/mL for 4 wk. The main findings of the study revealed that diabetic female rats treated with *L. fermentum* MCC2759 and MCC2760 reduced blood glucose levels, increased insulin levels, and improved the lipid profile[48]. Coupled with biochemical changes, *L. fermentum* administration downregulated TNF-α mRNA and up-regulated mRNA IL-10 in the intestine, liver, mesenteric adipose tissue, and muscle, suggesting that the anti-diabetic effect promoted by *L. fermentum* MCC2759 and MCC2760 can be associated with a decrease in inflammatory markers[48]. In addition, *L. fermentum* MCC2759 and MCC2760 administration modulated other gene expressions, such as reduced expression of Toll-like receptor 4, enhanced expression of tight junction protein ZO-1, endocannabinoid receptor CB2 and GLP1, glucose transporter type 4 in mesenteric adipose tissue and muscle tissue. The results demonstrated that *L. fermentum* MCC2759 and MCC2760 might be a potential probiotic in treating type 2 DM.

L. fermentum MF423

L. fermentum MF423 is a strain isolated from Chinese rice noodle wastewater[31]. The authors analyzed adverse effects triggered by an experimental model of type 2 DM induced by STZ and tested the effectiveness of different therapies, including supplementation with unfermented extracts of defatted rice bran, high and low doses of defatted rice bran fermented by *L. fermentum* MF423, and drug intervention (pioglitazone)[31]. Mice receiving a high dose (1 g/kg) of defatted rice bran fermentation extracts containing *L. fermentum* MF423 for 8 wk evidenced weight loss and reduced fasting blood glucose, lipid accumulation, and liver cells damage[31]. Moreover, probiotic groups intensified antioxidant activity in diabetic mice through up-regulation levels of SOD, total antioxidant capacity (T-AOC), and reversed elevation of malondialdehyde (MDA) in the liver[31]. It is important to mention that no effects were found in animals treated only by unfermented extracts of rice bran, highlighting the antioxidant activity of *L. fermentum* MF423.

To complete the evaluation of the therapeutic potential of *L. fermentum* MF423, the authors investigated the role of this probiotic in the modulation of GM. Diabetic rats treated either to high dose of defatted rice fermented by *L. fermentum* or pioglizatone showed GM composition similar to the control group, compared to untreated diabetic animals[31]. The relative abundances of *Bacteroidetes* (20%) and *Firmicutes* (40%) were increased in both mentioned groups compared to a diabetic group without treatment[31]. A decreased abundance of *Firmicutes* can be found in diabetic patients compared to their non-diabetic counterparts[53]. These two major phyla may play an essential role in hyperglycemia, hyperlipidemia, and inflammation. Moreover, probiotic treatment increased the relative abundance of SCFA-producing bacteria in diabetic mice, including *Lactobacillus, Parabacteroides, norank_f_Ruminococcaceae, Ruminococcus_torques*_group, and *Alloprevotella*. Interestingly, a decrease in the genus *Lactobacillus* was significant in diabetic mice, while treatment with defatted rice bran fermented by *L. fermentum* MF423 increased its abundance, similar to control mice[31]. *L. fermentum* is known for its probiotic role in food consumption, which could modify abnormalities in intestinal microbes and retard hyperglycemia. In conclusion, defatted rice bran fermentation by *L. fermentum* MF423 Lessened damage to the structure and function of GM induced by type 2 DM.

L. fermentum MTCC: 5898

Probiotic fermented milk prepared using different probiotic strains, including *L. rhamnosus* MTCC: 5957, *L. rhamnosus* MTCC: 5897, and *L. fermentum* MTCC: 5898, were evaluated in an experimental study[54]. Probiotic strains were offered independently or in combination for treating STZ induced type 1 DM in male *Wistar* rats. All probiotic strains were provided in a dosage of 1 × 10^9 CFU for 6 wk. The study demonstrated that the diabetic rats who received fermented milk containing *L. fermentum* MTCC: 5898 had less weight loss, improved glucose metabolism by reducing fasting blood glucose, HbA1c associated with increased insulin level, reduced diabetic dyslipidemia, and attenuated inflammation status through reduction of IL-6 and TNF-α[54]. In addition, supplementation with *L. fermentum* MTCC: 5898 showed antioxidant properties by increasing catalase (CAT) and SOD activities in the kidney and liver[54]. Moreover, administration of probiotics reduced mRNA expression of phosphoenolpyruvate carboxykinase and Glucose 6-phosphatase genes that code the key enzymes of the gluconeogenesis pathway[54]. Compared to other lactobacilli strains, rats receiving *L. fermentum* MTCC: 5898 displayed the most effective responses including oral glucose tolerance, serum insulin, serum, liver CAT, serum triglycerides, VLDL[54]. Therefore, it is suggested that daily consumption of probiotic fermented milk, especially *L. fermentum* MTCC: 5898, may be effective in attenuating complications of type 1 DM.

L. fermentum MTCC 5690 and MTCC 5689

L. fermentum MTCC 5690 and MTCC 5689 were isolated from the Indian gut and used to treat high-fat diet-induced type 2 DM mice[49]. The present study compared the anti-diabetic effect of *L. fermentum* MTCC 5690 and MTCC 5689 to other probiotics (*Lactobacillus rhamnosus*, *Lactobacillus plantarum* MTCC5690) and drug intervention (metformin, vildagliptin). *L. fermentum* MTCC 5690 and MTCC 5689 were administered in a concentration of 1.5 × 10^9 colonies/mouse/day for 24 wk[49]. Both probiotics and drugs groups reduced body weight, improved oral glucose tolerance, and reduced fasting glucose

and Hb1Ac levels in diabetic mice[49]. Concerning insulin levels, probiotic groups contributed to normalizing levels of this hormone, which approximated the levels observed in the control group[49]. In addition, a significant reduction of insulin levels was found in the vildagliptin group compared with other groups, which may be considered a possible adverse mild effect of this drug. Furthermore, all treatment groups improved lipid profile by reduction of levels of cholesterol, triglycerides, LDL, associated with an increase in HDL levels.

Additionally, the study evaluated the gut integrity after 24 wk of treatment. Probiotic treatment and drug therapy were able to reduce damage in gut integrity, which may contribute to normalizing gut permeability[49]. The authors quantified mRNA expression of epithelial tight junction occludin and ZO-1 and LPS levels. All the probiotic and anti-diabetic drugs increased gene expression of the intestinal tight junction occludin and ZO-1[49]. To evaluate endotoxemia state and intestinal barrier integrity, probiotic treatments decreased LPS levels and pro-inflammatory cytokines IL-6 and TNF-α in mice subjected to type 2 DM[49]. In addition, the authors verified the effectiveness of treatments in attenuating endoplasmic reticulum stress of skeletal muscle of diabetic mice. Results showed that both probiotic and drug therapies reduced endoplasmic reticulum stress markers[49]. The findings suggested that *L. fermentum* MTCC 5690 and MTCC 5689 act like anti-diabetic drugs, highlighting the therapeutic potential of these probiotic strains in alleviating type 2 DM complications.

Non mentioned strain of L. fermentum

The role of *L. fermentum* fruit extracts of *Syzygium cumini* and *Momordica charantia*, isolated from yogurt samples (Pakistan) on STZ induced DM mice, was previously investigated[55]. *L. fermentum* and the extracts were administered individually as well as in combination with DM-induced mice for 3 wk. Results were compared with mice that received drug intervention (Glucophage). Administration of probiotics and natural extracts improved body weight, and reduced blood glucose levels and both results were similar with the Glucophage group[55]. Concerning lipid profile, *L. fermentum* and natural extracts improved almost all lipid profile parameters, including reduced triglycerides, LDL, and increased HDL serum levels[55]. The study demonstrated that Glucophage treatment might affect some parameters, such as increased total cholesterol, triglycerides, and LDL concentration. These findings showed that *L. fermentum* and natural extracts have hypoglycemic and hypolipidemic activity, which may reduce DM complications.

Another experimental study demonstrated that mixed probiotics containing *L. fermentum* could reverse insulin resistance, reduce blood glucose levels, and improve lipid profile in STZ induced DM in old male Kunming mice after 4 wk of treatment[35]. In addition, the authors showed a significant impact of the supplementation of *L. fermentum* on the relief of gut dysbiosis, lowering the damage in the composition of GM[35]. However, the authors did not specify which strain of *L. fermentum* was administered, limiting the understanding of these effects.

Regarding clinical data, we found only one randomized, double-blind, placebo-controlled trial that evaluated the effectiveness of *L. fermentum* in attenuating complications of DM[30]. This study was carried out to assess the effects of probiotic supplementation on genetic and metabolic profiles in patients with gestational DM, aged 18-40 years (at weeks 24-28 of gestation)[30]. Participants were randomly divided into two groups: a control group and a probiotic group, made up of women who received a probiotic capsule containing *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum, L. fermentum* (2×10^9 CFU/g each) for 6 wk[30]. However, the authors did not inform the specific strain of *L. fermentum* used, limiting the interpretation of results. The probiotic group showed lower levels of fasting blood glucose serum insulin, reduced insulin resistance, and significantly increased insulin sensitivity compared with the control group[30]. In addition, probiotic supplementation decreased triglycerides, VLDL, and increased HDL levels compared with the control group. Additionally, probiotic administration reduced plasma MDA, and an elevation in plasma nitric oxide and T-AOC was found compared with the control group. Therefore, the probiotic treatment showed great therapeutic potential in alleviating complications found in women with gestational DM. Future clinical studies are needed to investigate further the specific strains of *L. fermentum* to elucidate which strains are more effective in attenuated DM.

CONCLUSION

This literature review showed that *L. fermentum* is a promising strain for the management of DM (Figure 1). Evidence from experimental and clinical study verified that *L. fermentum* supplementation contributed to normalizing body weight, reduced blood glucose and fasting blood glucose levels, reduced insulin resistance, and improved lipid profile. Coupled with these biochemical changes, *L. fermentum* therapy showed anti-oxidant and anti-inflammatory properties, which contributed to alleviating related symptoms of DM. However, the heterogeneities of studies, including variations in dosage, and duration of treatment, limit the elucidation of the most effective way to use *L. fermentum* as adjuvant therapy of DM. Moreover, it is relevant to explore the effectiveness of co-intervention with *L. fermentum* associated with bioactive compounds with antioxidant and anti-inflammatory properties,

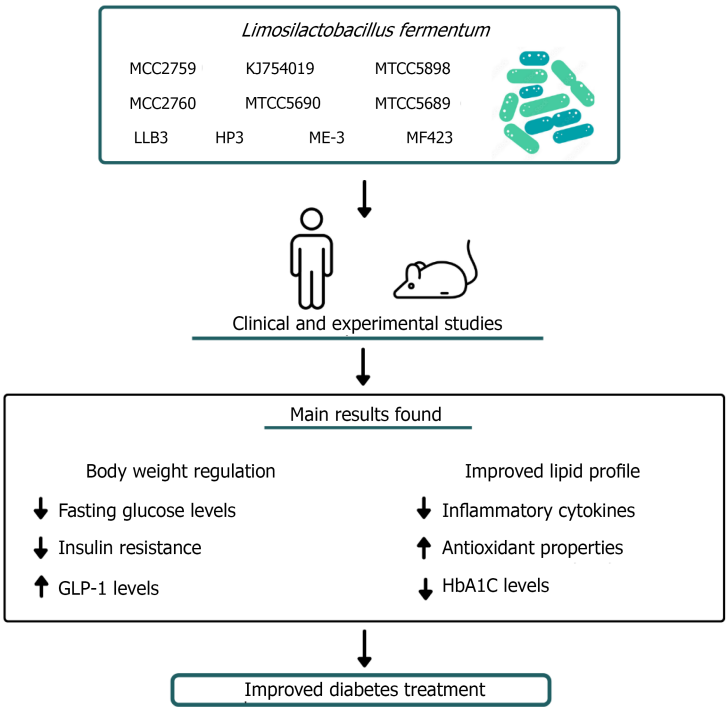

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Figure 1 Schematic drawing showing that *Limosilactobacillus fermentum* exert an anti-diabetic effect.

such as quercetin and resveratrol [15]. We also highlight that most of the available data came from preclinical studies, hence, therapeutic potential of different strains of *L. fermentum* in minimizing complications of DM needs to be further investigated in randomized, double-blind, placebo-controlled trials to confirm these findings in human studies.

ACKNOWLEDGEMENTS

The authors are grateful to the Fundação de Apoio à Pesquisa do Estado da Paraíba (FAPESQ, Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarships awarded to Lacerda DC, Trindade da Costa PC, Pontes PB, Carneiro dos Santos LA, Cruz Neto JPR, Silva Luis CC. Additionally, the authors give thanks for the research productivity fellowship granted by the Brazilian National Council for Scientifc and Technological Development (CNPq) to de Brito Alves, JL.

FOOTNOTES

Author contributions: de Brito Alves JL contributed to the conceptualization; Lacerda DC, Trindade da Costa PC, Pontes PB, Carneiro dos Santos LA, Cruz Neto JP, Silva Luis CC, de Sousa Brito VP, de Brito Alves JL wrote, reviewed and edited the manuscript.

Conflict-of-interest statement: The authors declare no competing interests.

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S-Editor: Chang KL
L-Editor: A

P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 729-737

DOI: 10.4239/wjd.v13.i9.729

ISSN 1948-9358 (online)

MINIREVIEWS

COVID-19 associated diabetes mellitus: A review

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Dasuqi SA, Saudi Arabia; Nalunkuma R, Uganda; Wang MK, China; Wang MK, China

Received: March 14, 2022
Peer-review started: March 14, 2022
First decision: May 11, 2022
Revised: May 20, 2022
Accepted: August 17, 2022
Article in press: August 17, 2022
Published online: September 15, 2022

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Abstract

A significantly higher rate of new-onset diabetes in many coronavirus disease 2019 (COVID-19) patients is a frequently observed phenomenon. The resultant hyperglycemia is known to influence the clinical outcome, thereby increasing the cost of treatment and stay in hospital. This will also affect the post-hospitalization recuperation. It has been observed that new-onset diabetes in COVID-19 patients is associated with considerable increase in morbidity and may be associated with increased mortality in some cases. This mini-review focuses on the possible causes to understand how COVID-19-related diabetes develops, various associated risk factors, and possible mechanism to understand the natural history of the disease process, clinical outcome, associated morbidities and various treatment options in the mana-gement of post COVID-19 diabetes. A literature search was performed in PubMed and other online database using appropriate keywords. A total of 80 articles were found, among which, 53 of the most relevant were evaluated/analyzed and relevant data were included. The studies show that patients who have had severe acute respiratory syndrome coronavirus 2 infection leading to development of COVID-19 may manifest not only with new-onset diabetes but also worsening of pre-existing diabetes. Cytopathic effect and autoimmune destruction of insulin-secreting pancreatic beta cells, cytokine storm during the active phase of infection causing impaired insulin secretion and resistance, drug-induced hyperglycemia, undetected pre-existing hyperglycemia/diabetic condition, and stress-induced impairment of glucose metabolism are some of the

possible potential mechanisms of COVID-19-associated new-onset diabetes mellitus. Many studies published in recent times have found a significantly higher rate of new-onset diabetes mellitus in many COVID-19 patients. Whether it is an inflammatory or immune-mediated response, direct effect of virus or combination of these is unclear. The resultant hyperglycemia is known to influence the clinical outcome and has been associated with considerable increase in morbidity and increased mortality in some cases.

Key Words: Coronavirus disease 2019; Coronavirus disease 2019 associated diabetes; Coronavirus disease 2019 related diabetes; Hyperglycemia in coronavirus disease 2019 patients; New-onset diabetes; Post-coronavirus disease 2019 diabetes

Core Tip: New-onset diabetes is one of the most important complications in patients recovering from coronavirus disease 2019 (COVID-19). This review is focused on different hypotheses that help with understanding of the disease process and suggest management protocols for COVID-19-associated diabetes mellitus.

Citation: Gavkare AM, Nanaware N, Rayate AS, Mumbre S, Nagoba BS. COVID-19 associated diabetes mellitus: A review. *World J Diabetes* 2022; 13(9): 729-737
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/729.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.729

INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was declared a global pandemic by the World Health Organization (WHO) in March 2020. It continues to spread worldwide with about 452201564 confirmed cases and 6029852 deaths to date[1].

The speed with which this deadly virus spreads leaves no place for doubt that, at some time, a significant proportion of the world's population will be affected. Therefore, it is a matter of great concern to study the interaction of COVID-19 with other commonly occurring medical conditions to anticipate and find out how they will interact with each other and to decide a protocol for their management. Laboratory reports in almost all critically ill patients show severe hyperglycemia as a common finding and this is often considered a marker of disease severity[2]. A literature search for studies carried out during the pandemic shows that COVID-19 is associated with hyperglycemia in people with and without known diabetes mellitus. Hence, now there is sufficient evidence to support the fact that SARS-CoV-2 infection causes a diabetogenic state in COVID-19 patients[3,4]. In this mini-review, an attempt has been made to understand how COVID-19 related diabetes develops, its pathogenesis, clinical presentation, outcome and management protocol of new-onset diabetes mellitus in COVID-19 patients.

DIABETOGENIC EFFECT OF SARS-CoV-2 INFECTION IN COVID-19

SARS-CoV-2 infection leading to a diabetogenic state in patients of COVID-19 is now a well-established fact. Different studies carried out in the earlier days of the pandemic support this fact and they report that many patients with SARS-CoV-2 infection were diagnosed with diabetes mellitus after COVID-19. It has been found that many patients presented with diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state and required higher units of insulin to normalize the blood sugar levels[4-6].

CAUSES/RISK FACTORS

The severity of hyperglycemic levels in confirmed cases of COVID-19 infection was found to be proportional to the severity of infection. This can be attributed to the involvement of one or more inter-related processes like stress response associated with severe illness, cytokine storm with elevated levels of inflammatory markers like interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP),

lactate dehydrogenase and ferritin. Overdoses of steroids, pancreatic beta-cell damage/destruction resulting in a combined effect of insulin resistance and insufficiency disturbing glucose homeostasis have been reported as important risk factors. Apart from this, increasing age, high body mass index (BMI) and family history of diabetes are independent risk factors[7]. To make the situation worse, strict disciplinary actions taken to break the chain of infection (such as repeated rotatory lockdowns) could also have had an adverse impact such as limited access to clinical care, healthy diet and opportunities to exercise[8].

POSSIBLE POTENTIAL MECHANISMS

COVID-19 due to SARS-CoV-2 infection may manifest not only as new-onset diabetes but also causes worsening of pre-existing diabetes. Considering the evolving nature of the COVID-19 pandemic, it is not yet clearly understood whether SARS-CoV-2 infection causes new-onset diabetes by mechanisms similar to those established in the pathogenesis of type 1 or type 2 diabetes mellitus, or whether this itself is an atypical form of diabetes[9]. Moreover, it has also not been established whether COVID-19 patients remain at higher risk for developing new-onset diabetes or related complications following viral clearance and recovery. The literature reveals detailed discussions regarding the possible potential mechanisms for derangement of glucose metabolism leading to the development of hyperglycemia and new-onset diabetes in COVID-19 patients and these can be broadly attributed to the following factors (Figure 1).

Cytopathic effect causing beta-cell damage

The entry portal for SARS-CoV-2 is angiotensin-converting enzyme (ACE)-2 receptor. Along with respiratory epithelial cells, ACE-2 receptors are also present in the kidneys, gastrointestinal tract and pancreas. Following infection, SARS-CoV-2 replicates in human endocrine and exocrine secretory cells of the pancreas[10]. It has been postulated that this causes the destruction of insulin-secreting pancreatic beta cells, which leads to the development of new-onset diabetes in some patients with COVID-19. This phenomenon can be well correlated with that observed during SARS-CoV-1 infection; thus, giving due credit to this hypothesis[11].

Autoimmune destruction of pancreatic beta cells

Apart from direct virus-induced cytotoxicity over insulin-secreting beta cells of the pancreas, another suggestion is that SARS-CoV-2 can trigger an autoimmune response against pancreatic beta-cell antigens, and it has emerged as one of the most prevalent hypotheses behind the etiopathogenesis of type 1 diabetes. According to this theory, the virus-mediated cytotoxicity toward beta cells leads to sequestration of antigens that in turn cause activation of autoreactive T lymphocytes. The resultant autoimmune response ultimately destroys the remainder of the beta-cell mass, leading to insulin-dependent type 1 diabetes in a few weeks to months after infection[12]. This theory cannot completely explain the pathogenesis of immediate onset of diabetes during the acute phase of COVID-19 infection; however, it may hold true for development of hyperglycemia in some patients and later development of diabetes within weeks to months post-COVID recovery. Further research about this would be helpful to reach a more meaningful conclusion.

Host response to COVID-19

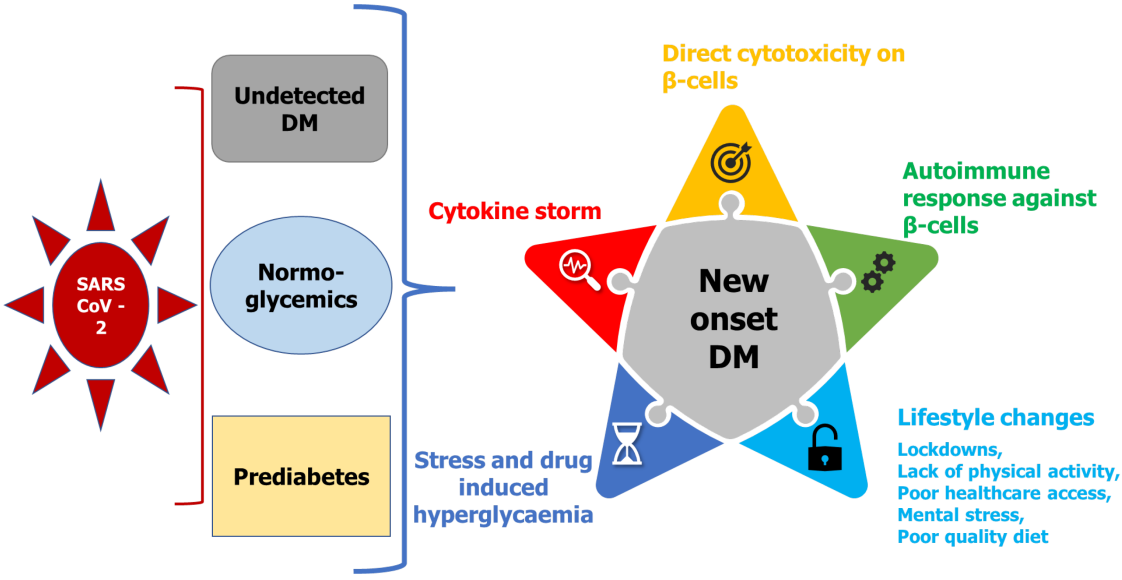
As observed in any acute infectious condition, a profound and nonspecific activation of immune mechanisms also occurs in patients with severe COVID-19, escalating the release of counter-regulatory hormones and proinflammatory cytokines such as IL-6 and TNF-α in the form of cytokine storm. This rampant cytokine storm is known to induce insulin resistance and resultant hyperglycemia[13].

Drug-induced iatrogenic effect

The RECOVERY trial in ICU COVID-19 patients requiring respiratory support prompted WHO to reframe guidelines and recommend the use of corticosteroids to reduce the overall mortality and morbidity in such patients[14]. However, corticosteroids are a double-edged sword. On one side, they improve the clinical course of patients during the cytokine storm and thereby prevent death in patients with COVID-19 pneumonia. On the other side, they are also known to be highly diabetogenic drugs. Hyperglycemia is almost inevitable with the doses prescribed for this indication and some cases present with complications like DKA, especially in patients with previously undiagnosed diabetes or prediabetes[8].

Undetected pre-existing diabetes before infection with SARS-CoV-2

The latest report of Diabetes Atlas from the International Diabetes Federation states that almost 50% of the adult population may have undiagnosed diabetes and bear a lifetime risk of diabetes mellitus[15]. This potential at-risk population is the reason for the hike in incidence of new-onset diabetes after COVID-19. Probable causes for this are recent weight gain, worsening of hyperglycemia due to changes



DOI: 10.4239/wjd.v13.i9.729

Figure 1 Possible potential mechanisms for development of post-coronavirus disease 2019 diabetes new onset diabetes. DM: Diabetes mellitus.

in lifestyle such as lack of exercise and reduced physical activity due to lockdown, self-isolation, social distancing and poor diet as a result of lack of access to sufficient quality and quantity of fruits/vegetables during lockdown periods[16].

Acute illness and stress leading to hyperglycemia and new-onset diabetes

Any acute illness and associated stress are the two important common factors that may lead to hyperglycemia in many patients and these patients will form the category of new-onset diabetes mellitus. This was observed during the SARS-CoV-1 pandemic[17]. The cytokine storm due to acute infection by SARS-CoV-2 can cause elevated inflammatory markers like an increase in CRP, erythrocyte sedimentation rate and increased leukocyte count. Cellular stress during acute inflammation causes accelerated lipolysis, thereby increasing the levels of free fatty acids in the circulation, leading to relative insulin deficiency[18].

New-onset diabetes has been reported in most of the earlier studies from different parts of the globe (Table 1)[4,19-30].

CLINICAL OUTCOME AND ASSOCIATED MORBIDITIES

It has been observed that diabetes is the pre-existing condition in most of the patients with COVID-19 disease showing severe morbidity and mortality[31]. The diabetic patients in general were found to have a higher risk of developing diabetic nephropathy, ischemic heart disease, and pneumonia leading to multiorgan failure and acute respiratory distress syndrome (ARDS) as compared to nondiabetic individuals. In addition, diabetic individuals were found to be more prone to ICU admission[32,33]. In subjects with diabetes and COVID-19, the mortality rate ranges from 22% to 31% of all COVID-19 patients[34]. A UK-based study revealed that out of 23 804 deaths in hospitalized COVID-19 patients, 32% had type 2 diabetes and 1.5% had type 1 diabetes mellitus[35].

Obesity, one of the independent risk factors for type 2 diabetes mellitus, is significantly associated with the severity of COVID-19. A cohort study of 2741 hospitalized patients found that among different factors, obesity was strongly associated with COVID-19 hospitalization and risk of critical illness[36]. A retrospective study from Kuwait consisting of 1158 hospitalized COVID-19 patients concluded that patients with morbid obesity needed more ICU admissions [odds ratio (OR), 5.18][37]. A study by Cai *et al*[38] involving 383 hospitalized COVID-19 patients reported that COVID-19 manifestations were more severe in obese patients as compared to patients with normal BMI. They also found an increased OR of developing severe COVID-19 in overweight patients (OR, 1.84; P = 0.05), with the value of odds being higher in obese subjects (OR, 3.40; P = 0.007).

Altered glucose homeostasis and insulin resistance resulting in acute hyperglycemia have been reported during infection in patients hospitalized with viral infections such as human herpes virus 8 and SARS-CoV as a part of normal antiviral responses. Such responses may further increase the risk of developing type 1 or type 2 diabetes mellitus[39]. During the SARS-CoV-1 outbreak in 2003, findings

Table 1 New-onset diabetes studies reported from different parts of globe

Ref.	Country	Study Design	Number of Cases	Results
Li *et al*[4]	China	Retrospective Observational	453	21 % were newly diagnosed with DM
Unsworth *et al* [19]	United Kingdom	Cross-sectional	33 children	30 children with new onset T1D
Ebekozien *et al* [20]	United States	Cross-sectional	64	6 cases with new onset T1D
Armeni *et al* [21]	United Kingdom	Case series	35	5.7 % cases newly presented with DM
Sathish *et al*[22]	China, Italy, United States	Systematic review	3711 cases from 8 studies	492 cases newly presented with DM
Wang *et al*[23]	China	Retrospective	605	176 cases newly detected with DM
Yang *et al*[24]	China	Retrospective Cohort	69	Prevalence: 53.85% in critical cases and 13.95% in moderately severe cases
Fadini *et al*[25]	Italy	Retrospective	413	5 % cases newly detected with DM
Wu *et al*[26]	Australia	Retrospective	8	Newly diagnosed cases showed C-peptide levels, negative anti-GAD antibodies consistent with T2D
Ghosh *et al*[27]	India	Retrospective Cohort	555	Higher levels of FBG, PPBG, HbA1c in newly diagnosed cases
Zhang *et al*[28]	China	Retrospective	312	Higher risk of adverse outcomes
Smith *et al*[29]	United States	Retrospective	184	6 patients showed elevated FBG
Liu *et al*[30]	China	Retrospective	233	Increased risk of in-hospital deaths

DM: Diabetes mellitus; FBG: Fasting blood glucose; GAD: Glutamic acid decarboxylase; HbA1c: Glycated hemoglobin; PPBG: Post-prandial blood glucose; T1D: Type 1 diabetes; T2D: Type 2 diabetes.

from one study involving 39 patients without a history of diabetes mellitus, showed that 20 patients developed diabetes during hospitalization and two of these patients remained diabetic despite receiving 3 years of antidiabetic management during follow-up[11].

In a study by Li *et al*[4], 94 out of 453 COVID-19 patients were diagnosed with new-onset diabetes. These newly diagnosed, post-COVID-19 diabetic patients required admission, intermittent mandatory ventilatory assistance, and demonstrated higher risk of all-cause mortality than those COVID-19 patients who were normoglycemic or had transient hyperglycemia. Also, these COVID-19 patients with pre-existing diabetes and new-onset diabetes demonstrated more severe complications including ARDS, acute renal failure, shock, or hypoalbuminemia as compared to those COVID-19 patients having normal or transiently raised blood sugar levels. Similarly, another multicenter retrospective study by Wang *et al* [23], involving 605 COVID-19 patients found that 29% of patients with newly detected diabetes mellitus experienced a higher rate of in-hospital complications and all-cause mortality as compared to normoglycemic COVID-19 patients over a 28-d period. Finally, another study by Fadini *et al*[25], comprising 413 subjects, reported a significant increase in ICU admissions and a higher percentage of death in patients with new-onset COVID-19-related diabetes compared to COVID-19 patients with pre-existing diabetes or normal blood glucose levels. In a retrospective observational study from Wuhan by Zhang *et al*[40], there was no significant increase in these parameters. Although many other studies have indicated a correlation between new-onset diabetes and COVID-19, experimental findings from several studies like Ibrahim *et al*[41] and Drucker[42] have also reported an inconclusive relationship between the increase in type 1 diabetes mellitus during the COVID-19 pandemic. These observations can be attributed to a lack of strong supporting evidence. Therefore, there is a necessity for further research to elucidate the interconnected relationship between COVID-19-induced diabetes mellitus and associated complications.

MANAGEMENT OF COVID-19-ASSOCIATED DIABETES MELLITUS

Since the explicit mechanisms and epidemiological factors associated with the development of new-onset diabetes following COVID-19 are unknown, it is difficult to frame treatment guidelines for such patients. However, in the light of increasing morbidity and mortality in people with newly diagnosed diabetes mellitus or those with hyperglycemia during admission, treatment protocols should prioritize

the management of acute hyperglycemia. It is also indispensable to diagnose COVID-19-associated diabetes mellitus and manage its metabolic complications such as DKA in patients admitted to the hospital for better clinical outcomes. Insulin requirement is invariably higher in such patients when compared to that in patients with acute illness due to other reasons or non-COVID-19-related DKA[26,43,44]. The exact duration of hospital stay of patients with newly detected diabetes mellitus following SARS-CoV-2 infection cannot be defined. There is a paucity of data in the literature regarding the long-term follow-up of these patients. Patients with stress-induced hyperglycemia may revert to a normoglycemic state once they have recovered from the phase of acute illness. Our experience also shows that most patients who have developed new-onset diabetes following SARS-CoV-2 infection have been found to revert to normoglycemic state within 2–4 wk after recovery, especially patients aged < 60 years. These patients, therefore, may not be labeled as having full-blown diabetes requiring prolonged antidiabetic medication. However, these cases are at high risk for developing diabetes in the future; therefore, they require long-term follow-up to determine a further course of action.

Recently, in a study report from India by Kuchay *et al*[45], three COVID-19 patients presented with acute-onset diabetes mellitus with DKA and had favorable initial response to treatment with intravenous fluids and insulin. Later, these patients were managed with multiple doses of subcutaneous insulin, and after 4–6 wk, they were shifted from insulin to oral hypoglycemic agents. Glutamic acid decarboxylase antibodies were measured in two patients who had tested negative, suggesting a transient insulinopenia in these patients.

Considering associated comorbidities like obesity, hypertension, hypercholesterolemia, coronary artery disease, renal disease, *etc.* in COVID-19 patients, hypoglycemic agents that improve metabolic function without weight gain should be the preferred choice for long-term management in patients following acute SARS-CoV-2 infection and sustained symptoms (*i.e.*, long COVID). Sodium–glucose co-transporter-2 (SGLT2) inhibitors and glucagon-like peptide-1 receptor agonists (GLP-1RAs) are the preferred novel therapeutic options that have a beneficial effect on factors like body weight, glycemic control, and cardiovascular and renal outcomes by reducing the duration of stay, overall morbidity and mortality from cardiac and noncardiac causes[46].

Therapeutic trials

DARE-19: This was a randomized, double-blind, placebo-controlled trial undertaken to study organ-protective effects of dapagliflozin, an SGLT-2 inhibitor. The study was conducted in hospitalized COVID-19 patients with at least one cardiometabolic risk factor (*i.e.*, hypertension, type 2 diabetes, coronary artery disease or chronic kidney disease). The trial excluded critically ill patients. The results showed that although the drug was well-tolerated by patients, it did not have an organ-protective effect. There was no significant improvement in clinical recovery within 30 d of starting the medication[47].

Ongoing trials: Various trials with dipeptidyl peptidase-4 inhibitors, pioglitazone, and the GLP-1RA semaglutide have been designed[48-53], but only a few are currently functional in the recruiting phase [52,53].

Long-term surveillance of COVID-19-associated newly diagnosed diabetes patients is necessary to control their risk factors and achieve adequate glycemic control. Patients with stress hyperglycemia during acute critical illness are at high risk of developing diabetes in the future. Meticulous tracking of such cases for early diagnosis, interventions, and long-term follow-up is necessary. Screening for diabetes in every COVID-19 patient would identify a significant number of cases and the cost-effectiveness of the screening would then need consideration. However, screening for diabetes is advisable at least for high-risk patients because if identified, appropriate management of these cases can be instituted. Also, COVID-19 patients with one or more comorbidities should undergo regular monitoring for cardiac and renal risk factors as well as micro/macrovascular complications.

CONCLUSION

The results of most of the earlier studies show that a significantly higher rate of new-onset diabetes in many COVID-19 patients is a frequently observed phenomenon. The resultant hyperglycemia is known to influence the clinical outcome and has been associated with considerable increase in morbidity and increased mortality in some cases. These issues increase the overall cost of treatment and the length of stay in hospital.

Hyperglycemia may return to normal glycemia in prediabetic or nondiabetic patients once they recover from acute illness and may not require antidiabetic medications. However, long-term follow-up is the key in such cases. Important prognostic factors include early diagnosis, associated other comorbidities, interventions, and longer surveillance of patients with stress hyperglycemia and/or new-onset diabetes so that we can ensure that their risk factors are managed and good glycemic control is achieved.

Studies published in recent times assessed the findings of hospitalized COVID-19 patients. There are no or limited data available from patients who were asymptomatic or had mild disease managed in community COVID care centers or in home isolation. So, there is likely to be a greater number of cases

of newly detected diabetes in COVID-19 patients worldwide. Hence, a large population of patients needs to be followed up globally to have better understanding of this phenomenon, involving an epidemiological and interventional approach.

ACKNOWLEDGMENTS

Authors wish to thank Mr. Vinod Jogdand and Mr. Dipak Badne from Dept. of Medical Education for their assistance in preparation of manuscript.

FOOTNOTES

Author contributions: Gavkare AM, Nanaware N and Rayate AS contributed to the literature search, collection of the data and writing the paper; Mumbre S and Nagoba BS contributed to the idea behind the manuscript, writing the paper, modification of content and final approval of the draft.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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S-Editor: Chang KL
L-Editor: Kerr C
P-Editor: Chang KL

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World Journal of Diabetes

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World J Diabetes 2022 September 15; 13(9): 738-751

DOI: 10.4239/wjd.v13.i9.738

ISSN 1948-9358 (online)

MINIREVIEWS

Effectiveness and safety of COVID-19 vaccines in patients with diabetes as a factor for vaccine hesitancy

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Casaca W, Brazil; Wong YP, Malaysia

Received: March 27, 2022
Peer-review started: March 27, 2022
First decision: May 11, 2022
Revised: June 6, 2022
Accepted: August 6, 2022
Article in press: August 6, 2022
Published online: September 15, 2022

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Abstract

Diabetes mellitus is one of the most common comorbid conditions encountered in patients with severe acute respiratory syndrome coronavirus 2 infection accompanied by significantly increased mortality, prolonged hospital stay, and requirement of invasive mechanical ventilation. This review aims to present the effectiveness and safety profile of available coronavirus disease 2019 (COVID-19) vaccines in people with diabetes as a potential cause of hesitancy for vaccination. Data from published research proves a robust immune response following immunization for COVID-19 in diabetic patients with substantial production of virus-neutralizing antibodies; however, the observed immune response was unequivocally weaker than that in individuals without diabetes. This observation was further enhanced by the findings that worse glycemic control was associated with more suppressed antibody production. In contrast, individuals with optimal glycemic control performed similarly to healthy controls. In addition to the need for strict glucose monitoring and adequate diabetes treatment, those findings

reinforce the concept of diabetes-induced secondary immune deficiency and necessitate the application of booster doses to diabetic patients with priority. Nevertheless, after vaccination, reported adverse events were not different from those in the general population. No increase in severe adverse events was documented. While single case reports detected transient increases in blood glucose post-vaccination, more extensive trials could not replicate such a relationship.

Key Words: COVID-19; COVID-19 vaccines; Diabetes; Vaccine effectiveness; Vaccine; Vaccine hesitancy

Core Tip: Diabetes mellitus is a crucial contributor to coronavirus disease 2019 (COVID-19). This review highlights published research on the effectiveness of vaccination against COVID-19 and related adverse events. Despite data of a notable decrease in the immune response to vaccination of diabetic patients, studies point out the importance of strict glycemic control to achieve adequate immunity against severe acute respiratory syndrome coronavirus 2 and the need to prioritize people with diabetes for the administration of booster doses. Regarding adverse events, none were increased in frequency in the diabetic population, except sporadic transient hyperglycemia observed post-vaccination.

Citation: Vasilev G, Kabakchieva P, Miteva D, Batselova H, Velikova T. Effectiveness and safety of COVID-19 vaccines in patients with diabetes as a factor for vaccine hesitancy. *World J Diabetes* 2022; 13(9): 738-751
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/738.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.738

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the novel coronavirus causing coronavirus disease 2019 (COVID-19) encountered since December 8, 2019 when the first cases of pneumonia of unknown origin etiology were described in Wuhan, Hubei Province, China[1]. Since then, the World Health Organization has reported more than 472 million confirmed cases of SARS-CoV-2 infection and more than 6 million deaths[2]. Closely related to SARS-CoV-2, the SARS-CoV pathogen causing a pandemic in 2002-2003 was also established to have originated in bats that probably serve as the natural reservoir host for those two viruses[3]. Another related betacoronavirus, the Middle East respiratory syndrome virus, is the cause of frequently emerging local outbreaks. It was first hypothesized that the Middle East respiratory syndrome coronavirus originated in bats. Still, it was then unanimously proved that the reservoir host was dromedary camels causing spillovers to humans[4]. Zoonotic transmission of novel coronaviruses to humans has been suggested to continue as more and more viruses are detected in bats and spill over to humans[5]. The fatality risk has been estimated to be 0.5% to 1.3% of all confirmed COVID-19 cases, with significantly higher rates in advanced age groups, reaching 18.4% in hospitalized patients over 80 years old[6,7].

COVID-19 AND DIABETES PATIENTS

Diabetes mellitus is a chronic metabolic disease characterized by impaired glucose metabolism and increased blood sugar levels as a result of absolute insulin deficiency due to autoimmune destruction of the pancreatic beta-cells (type 1 diabetes and latent autoimmune diabetes of adulthood) or impaired insulin action and a progressive loss of adequate β-cell insulin secretion due to insulin resistance in the target tissues (type 2 diabetes)[8]. Besides increased blood glucose levels, diabetes is associated with numerous chronic microvascular and macrovascular complications, determining diabetes as a cardiovascular disease[8]. Moreover, diabetes is associated with chronic low-grade inflammation[9] and an increased risk of thrombotic events[10], dyslipidemia[11], and metabolic syndrome[12]. Finally, it is a common disease that affects approximately 537 million adults. More than 240 million adults live with undiagnosed diabetes[13]. Collectively, these facts are a prerequisite for determining diabetes as a major risk factor for COVID-19[14]. It is considered that diabetic patients are a high-risk population with a calculated six-times greater risk for hospitalization and twelve-times greater risk for death than the healthy population[15]. Therefore, guidelines clearly state that patients with diabetes are strongly recommended to be vaccinated against COVID-19[16].

It is now widely accepted that diabetes, ischemic heart disease, hypertension, and cerebrovascular disease are the most common chronic comorbid conditions in people suffering from severe COVID-19

and requiring intensive care unit admission[17]. Many studies suggest that prolonged hyperglycemia is related to the increased frequency and severity of any infection, not just COVID-19. A matched cohort study among English primary care patients found that 6% of infection-related hospital admissions and 12% of SARS-CoV-2-related deaths were attributable to diabetes. The incidence rate ratios were highest for soft tissue, bone, and joint infections and sepsis[18].

A retrospective observational study of clinical outcomes in 1122 confirmed cases of COVID-19 found that the mortality rate in patients with diabetes and/or uncontrolled hyperglycemia was 28.8%, significantly higher compared to 6.2% in patients without diabetes or hyperglycemia. Furthermore, the median length of stay was also longer among discharged survivors in diabetic or hyperglycemic patients. Both findings imply the need for meticulous hyperglycemia management in hospitalized COVID-19 patients[19].

Both type 1 and type 2 diabetes mellitus were associated with worse rates of all-cause mortality owing to the COVID-19 pandemic in a large population-based cohort study encompassing more than 98% of primary care practice patients in England[20]. It identified a steep and sizable relationship between HbA1c values and death outcomes. Compared to individuals with optimal HbA1c values (6.5%-7.0%), those with HbA1c > 10% had a dramatically increased in-hospital all-cause mortality (hazard ratio: 2.23; 95% confidence interval: 1.50–3.30; $P < 0.0001$ in type 1 diabetes and hazard ratio: 1.61; 95% confidence interval: 1.47–1.77; $P < 0.0001$ in type 2 diabetes), independent from other risk factors[20].

Obesity, being strongly associated with diabetes mellitus type 2, has been investigated as a critical factor in the immune dysregulation that accompanies severe COVID-19. For example, in a French center, the relative risk for the need for invasive mechanical ventilation was seven-fold greater in patients with a body mass index > 30 kg/m^2 than those within the normal range of body mass index[21].

Similarly, it has been discovered that obesity alters the immune response to influenza infection and vaccination. Compared to vaccinated normal-weight individuals, vaccinated obese adults demonstrated double the relative risk for influenza or influenza-like infection, despite evidence of seroconversion. The hyperleptinemia and hyperinsulinemia accompanying the obese state contribute to T cell dysfunction, leading to impaired immune response[22].

Monocytes and macrophages appear to have a hallmark role in the dysregulated immune state of severe COVID-19 infections. Experimental data show that higher extracellular glucose concentrations promote sustained monocyte glycolysis, increased SARS-CoV-2 replication in antigen-presenting cells, and proinflammatory cytokine expression, leading to T-cell dysfunction and lung epithelial damage [23]. Therefore, it has been hypothesized that mitochondrial reactive oxygen species generation stimulated by SARS-CoV-2 leads to hypoxia-inducible factor alpha synthesis. Hypoxia-inducible factor alpha increased the expression of ACE2 (the entry point of SARS-CoV-2 into lung epithelial cells), interleukin-1β, tumor necrosis factor-α, interleukin-6, and interferons α, β, and λ in infected monocytes. Those findings suggest that hypoxia-inducible factor alpha is necessary to induce glycolysis and the consequent proinflammatory state of SARS-CoV-2-infected monocytes[23].

In addition to proinflammatory cytokines and coagulation factor modulation, SARS-CoV-2–induced reactive oxygen species production and viral activation of the renin-angiotensin system (leading to increased angiotensin II expression) lead to insulin resistance, hyperglycemia, and vascular endothelial injury, contributing to acute respiratory distress syndrome as well as cardiovascular, cerebrovascular thromboembolic complications, and disseminated intravascular coagulation[24].

ROUTINE VACCINES FOR DIABETES PATIENTS

Diabetes patients (both type 1 and type 2) are at an increased risk of significant complications from vaccine-preventable illnesses, including hospitalizations and death. Even properly managed diabetes could be associated with second immune deficiency and increased susceptibility to infections due to impaired cellular immune function. Diabetes patients are more likely to die from pneumonia, bacteremia, and meningitis. In line with this, immunization offers the most effective protection against vaccine-preventable illnesses. Therefore, in the next paragraph, we provide information about the routine and recommended vaccination of diabetes patients to emphasize the solid background behind the vaccines that can be used to improve patient and doctor confidence in the vaccines while decreasing vaccine hesitancy.

Moreover, vaccine side effects are often minor and resolve on their own. Severe adverse effects are quite uncommon. Given all the information above and the usually immunocompromised status of diabetes patients, many routine vaccines are officially recommended. For example, the National Health Service in Great Britain recommends the inactivated intramuscular vaccine against seasonal influenza for patients with diabetes types 1 and 2. This is because the risk of severe disease is higher for them than for people without diabetes[25].

The Centers for Disease Control and Prevention (CDC) gives the same recommendation according to seasonal influenza. All patients with diabetes from 6 mo are recommended for the inactivated intramuscular vaccine against the disease. The CDC does not recommend the live attenuated influenza

vaccine, also known as the nasal spray, for people with diabetes types 1 and 2[26].

A multicenter, randomized, and controlled study from the Republic of Korea demonstrated the safety and effectiveness of trivalent subunit inactivated intramuscular influenza vaccine, which contained an A/California/7/2009 (H1N1)-like strain, an A/Victoria/361/2011 (H3N2)-like strain, and a B/Brisbane/60/2008-like strain[27]. The World Health Organization recommended the strains during the 2012-2013 influenza season. The scientists observed similar results of seroprotection rates against the A/H3N2 and the B strains in the diabetic and non-diabetic groups. However, the diabetic group had significantly lower rates than those in the non-diabetic controls for the A/H1N1 strain. In both groups, 1 mo after vaccination, the geometric mean titers and seroprotection rates had increased dramatically for all three virus strains ($P < 0.001$)[27]. According to this study, 6 mo after vaccination, differences in the immunogenicity profiles between the diabetic and the non-diabetic groups were proven, with the seroprotection rate much lower in the elderly diabetes group than in the elderly control group. The safety of the trivalent subunit inactivated intramuscular influenza vaccine was established during the study, and all the participants confirmed that the vaccine was well tolerated. The post-vaccination reactions were mild to moderate, with tenderness at the injection site being the most frequent local reaction. In the diabetes group, 34.3% of the patients reported this local adverse event compared to 45.3% in the control group ($P < 0.001$). From the systemic reactions, myalgia was most reported, followed by tiredness, headache, malaise, chills, and arthralgia[27].

Another highly recommended vaccine for patients with diabetes is the vaccine against pneumococcal disease. The CDC recommends the pneumococcal vaccine for all children younger than 2 years and all adults 65 years or older. In addition, adults aged 19 through 64 are recommended for vaccination if they have chronic illnesses (including diabetes), human immunodeficiency virus/acquired immunodeficiency syndrome, or cancer or smoke cigarettes[28].

In a randomized controlled trial among elderly adults with comorbidities, the 13-valent pneumococcal conjugate vaccine showed significantly higher vaccine efficacy among subjects with diabetes mellitus[29].

A German retrospective cohort study proved the effectiveness of the 23-valent polysaccharide vaccine for invasive pneumococcal disease provoked by *Streptococcus pneumoniae* 22/23 serotypes. Therefore, scientists have recommended increasing the vaccination coverage of 23-valent polysaccharide vaccine among elderly adults in Germany[30].

Herpes zoster is an infection that occurs after reactivation of the varicella-zoster virus and is most common in people older than 50 years who have age-related fading of the immune function and concomitant comorbidities[31]. Herpes zoster is more prevalent among people with diabetes mellitus [32]. There are currently two vaccines against herpes zoster, a live-attenuated vaccine and a recombinant zoster vaccine. The effectiveness of both vaccines resulted in a significant decrease in the incidence of the disease in the older adult population[33-35].

Regarding immune responses after vaccination, diabetes patients mounted an adequate B-cell immune response after influenza and the 23-valent polysaccharide vaccine[36]. However, they had a lower response to the hepatitis B vaccine[37]. All findings support the notion that vaccines for vaccine-preventable illnesses should be administered in a timely manner to diabetic patients, given that this population are susceptible to infection and have a higher risk of diabetes deterioration during infections.

COVID-19 VACCINES, AUTOIMMUNITY, AND GLUCOSE METABOLISM

Autoimmune inflammatory diseases are characterized by an abnormal immune response to self antigens[38]. The interactions between people with autoimmune diseases and SARS-CoV-2 infection are generally unexpected. The mechanisms underlying the possible complications and fatal outcomes are not fully understood. COVID-19, like other viral infections, has the potential to cause a flare, including in diabetes patients[39,40].

Although preliminary findings revealed that autoimmune diseases did not enhance the incidence of SARS-CoV-2 infection and severe disease[41], autoimmune disorders are associated with organ damage, chronic cardiovascular, metabolic and respiratory comorbidities, susceptibility to bacterial infections, and sometimes, B cell depletion therapy and usage of high-dose glucocorticoids. All these factors may enhance the risk of a poor prognosis for patients during the COVID-19 course[42]. As a result, COVID-19 preventive methods should focus on the unique group of autoimmune disease patients, with immunization against SARS-CoV-2 being one of the most promising approaches. Nonetheless, because of growing findings, their safety and efficacy should be primarily and regularly assessed in different patient populations, including patients with diabetes[43].

We will focus on vaccine hesitancy in patients with diabetes later. Still, aside from the fear of immunization-related autoimmunity, there is considerable evidence that the development of autoimmune diseases is influenced by a variety of other variables. In fact, because autoimmune illnesses can develop without immunizations, it is impossible to conclude that vaccines alone induce autoimmunity[44].

Also, people with autoimmune disorders are most concerned about whether the risk of disease flare or aggravation increases following immunization. However, more than 5000 studies confirmed that those with autoimmune illnesses were not at risk of aggravation or worsening conditions[45].

The approved COVID-19 vaccines have played the most significant role in the battle with the SARS-CoV-2 virus to reduce disease severity and mortality among those affected, especially those with diabetes. As of March 16, 2022, more than 10 billion doses of different COVID-19 vaccines have been administered worldwide, including booster doses[46]. So far, we know that although the COVID-19 vaccinations' immunogenicity and efficacy in the autoimmune disease patient population may be lower than in healthy controls, they are typically comparable. Furthermore, data on the vaccines' effectiveness in the autoimmune disease population of adults and children are lacking since only a few studies follow up on the duration of protection and different modalities of immune responses after immunization[47].

Vaccination is recommended as a priority for people with diabetes. The aim is to elicit a sustained immune response in the target population. There is evidence that glycemic control in diabetes significantly affects the immune response[48]. Therefore, it is important to determine whether glycemic disturbances occur before or after vaccination against COVID-19 in people with diabetes.

Monitoring blood glucose levels became critical during the COVID-19 pandemic because the data show two to three times higher hospitalizations and double the mortality rate among patients with simultaneous diabetes and COVID-19[49-51]. It also turned out that emerging diabetes, hyperosmolar hyperglycemic syndrome, and diabetic ketoacidosis could accompany post-COVID syndrome[52,53].

Very few studies have been conducted on how vaccination affects blood sugar levels. However, some effects of COVID-19 on glycolytic metabolism are already known[54]. Several cases of hyperglycemia followed by vaccination against COVID-19 were reported[53,55,56]. One diabetic woman and two diabetic men had post-vaccine hyperglycemia within 6 d of receiving the first dose of the Covishield vaccine (AstraZeneca). Hyperglycemia passed after about a month in the woman after treatment with a higher dose of metformin. At the same time, the two men achieved glycemic control in 3-15 d without an additional medication[56]. However, no association between vaccination and disturbed glycemic control was proven.

Another study reported hyperglycemia between the 20th and 36th days after the first dose of the AstraZeneca vaccine[55]. Similar conditions have also been reported following mRNA vaccines, Comirnaty (Pfizer/BioNTech) and Spikevax (Moderna)[53]. This case report demonstrated remarkably high blood sugar and HbA1c levels after vaccination in a patient with previously reasonable blood glucose control. However, this patient probably had undiagnosed diabetes since his two parents had type 2 diabetes and the patient himself had a clinical picture of insulin resistance[53,57].

Another retrospective study examined 96 adults over the age of 18 with type 1 diabetes before and after their first COVID-19 vaccination[58]. Fifty-nine percent of them had a significant deviation in blood glucose levels, which were controlled within 7-10 d after vaccination. Again, the data show no difference in the effects between the AstraZeneca and Pfizer vaccines.

There could be many reasons for fluctuations in blood glucose. Regarding existing studies, no excipients and/or adjuvants to vaccines have been reported to cause hyperglycemia, so that the condition could be related to the antigens in the vaccine against COVID-19. A possible mechanism for its occurrence is stimulating the immune system, which leads to a stress response. However, it is milder than usually occurs with COVID-19 infection. Different changes in the glycolytic pathway occur in COVID-19 infection in response to stress and lead to increased glucose levels in cells[54,59]. Stress also increases hormones such as adrenaline, cortisol, and/or glucagon that cause metabolism changes[60]. In addition, they affect the immune system by reducing the activity and number of natural killer cells and lymphocytes, decreasing antibodies and reactivating latent viral infections[61].

More research and patient results need to be analyzed to provide a clear and definitive answer about this temporary instability of blood glucose levels after the COVID-19 vaccination. Understanding changes in the glycolytic pathway associated with COVID-19 and/or after vaccination could help find a new treatment for this disease.

Clinical data support a strong response of the neutralizing antibodies in patients with diabetes after COVID-19[36]. However, patients should be consulted and prepared for possible hyperglycemia after the COVID-19 vaccination[60].

There is still no data on the effects on glucose levels after the second COVID-19 vaccination or booster dose. These studies are underway. The question remains if the immunity to vaccination against COVID-19 in people with diabetes would change or decrease.

COVID-19 VACCINES: DATA ON EFFECTIVENESS IN DIABETES PATIENTS

Although there is a high incidence of diabetes among populations, during the COVID-19 vaccine studies, patients with diabetes are usually rolled out. Therefore, we rely on the data from real-life studies from vaccinations after the vaccine approval.

Soetedjo *et al*[62], in their systematic review, managed to cover eight studies with a total of 64468 patients and 5156 patients with diabetes[62]. The vaccines included were BNT162b2 vaccine

(Pfizer/BioNTech), CoronaVac (Sinovac Life Sciences), Covishield™ (ChAdOx1-nCOV), and Covaxin™ (BBV-152). The effectiveness studies showed lower seropositivity and antibody responses following vaccination in diabetes patients than in healthy controls. This was observed from 1-4 wk after full COVID-19 vaccination.

The studies on the immunogenicity of COVID-19 patients with diabetes are presented in Table 1[63-72].

We can assume from the data that the seroconversion rates in diabetes patients following COVID-19 vaccination is lower, including lower antibody titers. However, the underlying reasons for that are not fully understood. It was proposed that impaired adaptive immune response in diabetes patients contributes to altered vaccination response[37]. Additionally, patients with diabetes had some immune alterations such as reduced circulating CD4+ cells, lymphocyte proliferation, and antigen presentation [67]. Immunological alterations in patients with diabetes are shown in Figure 1.

As we stated above, hyperglycemia at immunization may reduce the immune response. As a result, having sufficient glycemic control during the post-vaccination interval increases immunological response because strict glycemic control may predispose to a favorable immune response to the SARS-CoV-2 vaccine[67]. The host's ability to respond to infections and the formation of long-term immunological memory, including correct responses to immunizations, are both influenced by the immune system's steady degradation. Among other things, the adaptive immune system can be compromised by poor proliferation in response to antigenic stimulation, impaired generation of CD4+ T follicular helper cells, and a reduced capacity to generate effector lymphokines. Additionally, it is well-known that hyperglycemia induced glycosylated receptors on the immune cells lead to impaired immune cell function[67]. Immunological features and alterations of diabetes are shown in Figure 1.

In line with this, the leading cause of reduced immune response and protection after vaccination in diabetes patients remains relative immune deficiency. Other factors, such as poorly controlled diabetes, may indirectly impact the vaccines' efficacy and effectiveness. Thus, we must pay attention to hyperglycemia, which can influence clinical COVID-19 results and vaccination efficacy. This leads us to assume that maintaining proper glycemic control after immunization increases immunological response. Also, we anticipate that strict glycemic management will support the favorable immunological response to the SARS-CoV-2 vaccination. Therefore, glycemic management should be the standard during pandemics, which strengthens the role of diabetologists in vaccination program effectiveness[67].

Additionally, different vaccines elicited comparable results, as shown in Table 1. This is also valid for the adverse effects demonstrated in the next section of the paper.

To sum up, the vaccines' overall effectiveness could also be evaluated by the re-infection rate among patients with diabetes who were immunized against SARS-CoV-2. Generally, patients with diabetes were among the population of people with a higher risk of re-infection, both after natural infection or vaccination[73]. However, no particular numbers or percentages were cited for the re-infection rate after COVID-19 vaccination in diabetes patients, although the risk ratio for hospitalization due to re-infection was declared at 1.6[74]. The re-infection was less likely to occur in naturally infected or vaccinated people than in naïve patients. Therefore, the disease course was expected to be less severe in vaccinated diabetes patients.

COVID-19 VACCINES: DATA ON ADVERSE EFFECTS IN DIABETES PATIENTS

Although the benefits of vaccination against COVID-19 in diabetic patients are undeniable, we will try to systematize the information gathered in the literature on the side effects of COVID-19 vaccines. A recent study analyzing the side effects of the two mRNA COVID-19 vaccines (BNT162b2 mRNA and mRNA-1273) among 1245 healthcare workers described general and organ-specific symptoms after the first and/or second dose of mRNA vaccines in the United States. The common endocrine symptoms were decreased appetite (5.73%), heat/cold intolerance (3.24%), increased thirst (1.12%), increased appetite (0.87%), and increased urine production (0.25%)[75]. Importantly, there are no reported symptoms associated with glucose metabolism; nevertheless, there is no information about diabetic participants in this study. Commonly reported symptoms were soreness, fatigue, myalgia, headache, chills, fever, joint pain, nausea, muscle spasm, sweating, dizziness, flushing, feeling of relief, brain fogging, anorexia, localized swelling, decreased sleep quality, itching, tingling, diarrhea, nasal stuffiness, and palpitations. Despite this extended list of symptoms, 79.7% of participants did not violate daily activities. In comparison, around 98.0% of them planned to have the second dose, and 92.9% had already received it[75].

An anaphylactic reaction is another reported side effect post-vaccination. The CDC reported in January 2021 that anaphylaxis might occur more frequently after the BNT162b2 mRNA vaccine than other vaccines[76]. According to this report, 11.1 per million was the estimated rate of anaphylaxis from 1893360 first doses of the Pfizer-BioNTech COVID-19 vaccine. The total reported adverse events after vaccination were 4393 (0.2%). Of them, only 175 cases were identified as potentially life-threatening allergic reactions, and 21 were reported as anaphylaxis. Most of the observed allergic reactions have

Table 1 Existing studies on effectiveness of coronavirus disease 2019 vaccines in patients with diabetes

Ref.	Type of study	Type and name of the vaccine	Participants	Efficacy/effectiveness	Adverse effects
Nomura *et al*[63]	Observational study	BNT162b2	12 from a total of 252, at a mean age of 43.9 yr	Lower antibody titers compared to non-diabetic subjects 3 mo post-vaccination	N/A
Lustig *et al* [64]	Longitudinal cohort study	BNT162b2	139 from a total of 2498, at a mean age of 47.7 yr; mostly healthcare workers	Substantial antibody response after 2 doses, but overall lower concentrations of IgG and IgA in diabetics compared to healthy adults	N/A
Van Praet *et al*[65]	Case-control study	BNT162b2	25 from a total of 75, at a mean age of 85 yr	Decreased cellular immune response only in individuals with diabetes or active malignancy in the studied population	N/A
Ali *et al*[66]	Cohort study	BNT162b2	81	The BNT162b2 vaccine induced robust IgG and neutralizing antibody responses in people with and without T2DM. On average, diabetics had 13.86 BAU/mL less IgG and 4.42% less neutralizing antibodies compared to non-diabetics	N/A
Marfella *et al*[67]	Prospective observational study	BNT162b2, mRNA-1273, ChAdOx1-S	251, of which 134 with optimal glycemic control and 117 with poor glycemic control	21 d after the second dose, neutralizing antibody titers and CD4 Th1 cytokine responses were weaker in individuals with HbA1c > 7% compared to those with HbA1c < 7% whose titers were indistinguishable from those of healthy subjects	N/A
Singh *et al* [68]	Cross-sectional study	ChAdOx1-nCOV (Covishield), BBV-152 (Covaxin)	52 from a total of 463 at a mean age of 44.8 yr	Amongst all studied comorbidities, people with T2DM had lower seropositivity rates compared to those without (84.6% *vs* 96.1%)	N/A
Sauré *et al* [69]	Surveillance study	CoronaVac, BNT162b2	4626 from a total of 59987 people from Chile's population	IgG seropositivity was significantly lower in diabetics receiving the CoronaVac vaccine compared to healthy subjects	N/A
Piccini *et al* [70]	Retrospective cohort study	mRNA-1273BNT162b2	39	In adolescents and young adults with T1DM, vaccination with either product was safe and did not influence glycemic control	No serious adverse events were reported
Watanabe *et al*[71]	Observational study	Pfizer/BioNTech BNT162b2 vaccine	2 from a total of 66 at a mean age of 29 yr; mostly healthcare workers	Undetectable titers of anti-SARS-CoV-2 antibodies	N/A
Karamese and Tutuncu[72]	Cross-sectional study	CoronaVac	49 from a total of 186 people, at a mean age of 70.4 yr	Significantly lower levels of anti-SARS-CoV-2 antibodies in diabetes patients than in the controls	N/A

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IgG: Immunoglobulin G; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; N/A: Not applicable; BAU: Binding antibody units.

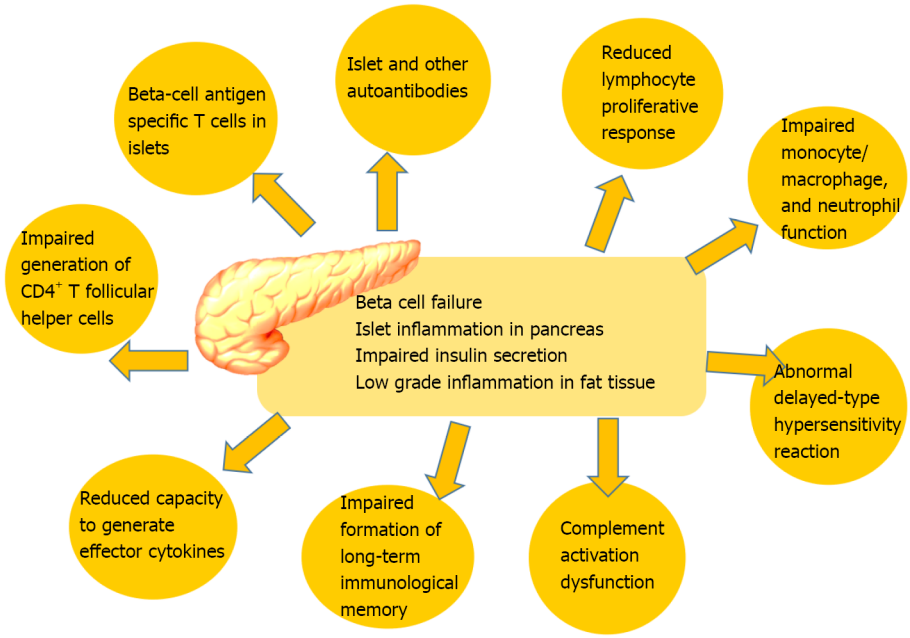
manifested within the first 30 min of vaccination. Anaphylaxis usually occurs in individuals with a history of allergies or a previous anaphylactic episode. Again, there is no information related to glucose disturbances or predisposition for allergic reactions among diabetes patients[76].

Another CDC report on the side effects of the two mRNA vaccines showed that the most frequently reported symptoms after vaccination were headache, fatigue, and dizziness. The rate of anaphylaxis was defined as rare (4.5 reported cases per million doses administered). There were no data on side effects associated with glucose metabolism and no evidence that vaccine symptoms were more pronounced in diabetics[77].

Increased risk of myocarditis and pericarditis has been reported after mRNA COVID-19 vaccination (Pfizer-BioNTech and Moderna)[78-82] and rarely after adenovirus vector-related vaccine[83,84]. Detailed analyses of these cases showed that myocarditis and pericarditis were more often in adolescents and young adult males. In addition, they were associated with multiple comorbidities, including obesity and hyperlipidemia[85].

An Italian study reported that the most frequent adverse events observed post-vaccination were vagal response (30%), anxiety reaction (24%), and dizziness (21%) among a total of 314671 vaccinated subjects. These side effects were predominantly observed in women and people with comorbidities; however, it is unclear whether diabetes was included[86].

Another study analyzed the adverse effects among 447346 reports 2 wk after vaccination with one of following three COVID-19 vaccines: 19462 Ad26.COV2.S (Janssen COVID-19 vaccine), 120580 mRNA-1273 (Moderna COVID-19 vaccine), and 100752 BNT162b2 (Pfizer-BioNTech COVID-19 vaccine). Headache, joint-related symptoms, muscle pain, musculoskeletal and connective tissue pain, nausea or



DOI: 10.4239/wjd.v13.i9.738

Figure 1 Immunological alterations in diabetes patients.

vomiting, dermal and epidermal conditions, and febrile disorders were common post-vaccination complaints. They were associated with delayed recovery in people with underlying diseases, including diabetes[36].

Theoretically, vaccination could be followed by mild to moderate elevation of blood glucose levels [87]. However, a few studies have already reported worsened glucose control after the COVID-19 vaccination[53,56]. However, a recent study showed that COVID-19 vaccination was not associated with impairment in glucose management. The study analyzed the short-term effects of COVID-19 immunization in patients with type 1 and type 2 diabetes who were vaccinated with one of the following three COVID-19 vaccines: BNT162b2, mRNA-1273, and AZD1222 (Oxford-AstraZeneca). The study collected and analyzed 49200 continuous glucose monitoring data from 74 participants in the study and showed that there were no changes in time spent in the target glycemic range (70−180 mg/dL) on the days of follow-up (2 d before and 3 d after vaccination). However, patients with type 1 diabetes and more pronounced post-vaccine side effects spent more time above the target range (> 180 mg/dL); no such observations were found in patients with type 2 diabetes. Additionally, the study reported no need for adjustment in the insulin bolus dose and changes in carbohydrate intake around the vaccination in patients with both diabetes types[88].

To sum up, the most common systemic side effects are headache, chills, fever, flu-like symptoms, nausea, and fatigue. The local effects are pain, redness, and swelling at the injection site on the arm. Patients with diabetes are not more prone to have pronounced side effects of COVID-19 vaccination than healthy people. However, most of them are mild and disappear a few days after vaccine administration. Although it is possible to have increased blood sugar levels after vaccination, it is rather not associated with a significant impact on glycemic control. Therefore, it does not require any changes in diabetic therapy. However, we must remember that COVID-19 may exert deteriorating effects in patients with autoimmune diseases[89], including type 1 diabetes. On the other hand, therapies for type 2 diabetes that target cytokines can also change the course of infection[90].

VACCINE HESITANCY IN DIABETES PATIENTS

Diabetes patients were not excluded from the COVID-19 vaccine trials due to the higher prevalence of the disease amongst the populations. Thus, we obtained much more data on the evidence for the long-term safety and efficacy of the COVID-19 vaccine in patients with diabetes, in contrast to other autoimmune diseases where many gaps in the knowledge still exist. However, despite the considerable information, physicians and patients still fear exacerbation of the disease and the adverse effects of vaccination, which increases the hesitancy to vaccination.

Even though COVID-19 vaccination has emerged as the sole practical approach to improving clinical outcomes, vaccine hesitancy remains a barrier to obtaining significant levels of vaccine coverage. This poses a particular concern to individuals suffering from autoimmune illnesses, who are already at a

higher risk of hospitalization and poor clinical outcomes due to COVID-19 infection. While long-term safety and effectiveness data for COVID-19 immunization in individuals with autoimmune illnesses are lacking, existing research clearly shows that the advantages of vaccination exceed the risks of side effects and disease flare-ups.

The COVAD study group demonstrated some causes for vaccination hesitancy, which was reported in around half of the patients with autoimmune illnesses in the study's pilot findings[91]. Of all the respondents who did not receive any dose of the COVID-19 vaccine, 16.94% reported not getting the vaccine due to long-term safety concerns or other fears, such as disease exacerbation and delayed adverse effects, and 27.45% stated that they plan to wait until more data are available on the safety of the vaccine before vaccination. Other reasons given by the respondents for not vaccinating are the lack of the vaccine in some parts of the world (32.00%), planning for vaccination at a later date (11.67%), and postponing vaccination due to recent COVID-19 infection (7.30%). Some patients also reported not getting the vaccine because they had been advised to do so by their doctor (5.40%)[91].

However, there are no medical recommendations against vaccination. Only 35% of those vaccinated had mild side effects (fever/headache/myalgia). Furthermore, patients with autoimmune diseases had fewer side effects than healthy controls. Recent international studies show a negligible risk of severe side effects or disease exacerbation after vaccination[91].

Wang *et al*[91] showed that more than half of 483 Chinese diabetes patients experienced vaccine hesitancy (58.2%). Of them, 41.8% were unwilling to get the COVID-19 vaccine. Although patients were aware of the severity of COVID-19 in diabetes patients, they were concerned about vaccine safety. Interestingly, the vaccination status of their relatives did not influence the patients' decisions, but disagreement with their physician on the ability of the vaccine to reduce the severity of COVID-19 correlated with vaccine hesitancy[92].

The five factors associated with vaccine hesitancy in diabetes patients are the false belief that diabetes is not a high risk factor for severe COVID-19 and a lack of confidence in vaccine efficacy to prevent infection. However, diabetes patients were convinced that diabetes worsens COVID-19 prognosis and that vaccination may reduce the transmission risk. The third factor associated with vaccine hesitancy was the fear of adverse effects, and the fourth and fifth were the dependence on the opinion of others, including vaccines to be administered to a large group of people, and the influence of social media on them[92].

Similarly, Aldossari *et al*[92] showed that 34.7% of Saudi diabetes patients in the survey were willing to be vaccinated, and 79.0% supported COVID-19 vaccination. However, they showed signs of fear and uncertainty[92]. Therefore, the key to a successful vaccination campaign for these patients remains the accurate information provided and the fight against misinformation. Some factors related to vaccine hesitancy were relatively quick development, beliefs that the trials were insufficient, fears and uncertainty of components, and especially the mRNA behavior after vaccination. In addition, an enormous impact is the anti-vaccination movements in social and traditional media. Furthermore, social media misinformation has led to increased anxiety and vaccine hesitancy.

CONCLUSION

Since diabetes mellitus is a significant contributor to COVID-19 mortality, patients with disturbed glucose metabolism should be protected from SARS-CoV-2 infection. Few studies have established data on the effectiveness and safety of the COVID-19 vaccine in patients with diabetes. Despite the significant reduction in the immune response to vaccination in diabetes individuals, they should be prioritized for complete vaccination and booster dose delivery. Also, glycemic management in achieving sufficient immunity against SARS-CoV-2 is mandatory. Data also showed that COVID-19 vaccines presented an excellent safety profile with adverse effects following vaccination similar to the healthy population and no increase in the incidence of adverse events in the diabetic group. Finally, vaccine hesitancy among diabetes patients could be overcome with proper information and patient care.

FOOTNOTES

Author contributions: Velikova T and Vasilev G contributed to the conceptualization; Vasilev G, Kabakchieva P, Miteva D, Batselova H, and Velikova T contributed to the investigation; Miteva D and Kabakchieva P contributed to the resources; Vasilev G and Miteva D wrote the original draft; Kabakchieva P, Batselova H and Velikova T wrote and editing the manuscript; Velikova T contributed to the supervision; all authors revised and approved the final version of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 752-764

DOI: 10.4239/wjd.v13.i9.752

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Balbaa ME, Egypt; Cheng JT, Taiwan; Zan-Chao L, China

Received: March 11, 2022
Peer-review started: March 11, 2022
First decision: April 25, 2022
Revised: May 13, 2022
Accepted: August 22, 2022
Article in press: August 22, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Benzylamine (Bza) oral administration delays the onset of hyperglycemia in insulin-resistant *db*$^{-/-}$ mice; a genetic model of obesity and type 2 diabetes.

AIM

To extend the antihyperglycemic properties of oral benzylamine to a model of insulin-deficient type 1 diabetes.

METHODS

Male Swiss mice were rendered diabetic by streptozotocin treatment (STZ) and divided in two groups: one received 0.5% Bza as drinking solution for 24 d (STZ Bza-drinking) while the other was drinking water *ad libitum*. Similar groups were constituted in age-matched, nondiabetic mice. Food intake, liquid intake, body weight gain and nonfasting blood glucose levels were followed during treatment. At the end of treatment, fasted glycemia, liver and white adipose tissue (WAT) mass were measured, while glucose uptake assays were performed in adipocytes.

RESULTS

STZ diabetic mice presented typical features of insulin-deficient diabetes: reduced body mass and increased blood glucose levels. These altered parameters were not normalized in the Bza-drinking group in spite of restored food and water intake.

Bza consumption could not reverse the severe fat depot atrophy of STZ diabetic mice. In the nondiabetic mice, no difference was found between control and Bza-drinking mice for any parameter. In isolated adipocytes, hexose uptake was partially activated by 0.1 mmol/L Bza in a manner that was obliterated *in vitro* by the amine oxidase inhibitor phenelzine and that remained unchanged after Bza supplementation. Oxidation of 0.1 mmol/L Bza in WAT was lower in STZ diabetic than in normoglycemic mice.

CONCLUSION

Bza supplementation could not normalize the altered glucose handling of STZ diabetic mice with severe WAT atrophy. Consequently, its antidiabetic potential in obese and diabetic rodents does not apply to lipoatrophic type 1 diabetic mice.

Key Words: Diabetes; Adipocytes; Amine oxidases; Insulin-like agents; Glucose transport; Polydipsia

Core Tip: In adipocytes, benzylamine (Bza) is oxidized by amine oxidases and stimulates glucose uptake. Bza oral administration alleviates insulin-resistant diabetes in obese and diabetic mice. It was investigated whether Bza was also antihyperglycemic in insulin-deficient type 1 diabetes. To this aim, a 0.5% Bza drinking solution was given to streptozotocin-induced diabetic mice. Oral Bza did not recover hyperglycemia and reduced adiposity of lipoatrophic and diabetic mice. A minimal level of adiposity was required to support benzylamine oxidation and to improve glucose utilization. Thus, the antidiabetic properties of Bza in obese and diabetic models, do not apply for diabetes with severe lipoatrophy.

Citation: Carpéné C, Stiliyanov Atanasov K, Les F, Mercader Barcelo J. Hyperglycemia and reduced adiposity of streptozotocin-induced diabetic mice are not alleviated by oral benzylamine supplementation. *World J Diabetes* 2022; 13(9): 752-764

URL: https://www.wjgnet.com/1948-9358/full/v13/i9/752.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i9.752

INTRODUCTION

A recent study indicates that orally given benzylamine (Bza) delays the onset of diabetes in obese and insulin-resistant $db^{-/-}$ mice[1]. Supplementation with 0.5% Bza (5 g/L) in the drinking water impaired the increase in blood glucose, water intake and urine emission that occurs after weaning in this mouse model of insulin-resistant type 2 diabetes. The proposed mechanism of action for ingested Bza, which is naturally present in vegetables and edible plants, relies on its oxidation by an amine oxidase, which is a copper-containing enzyme highly expressed in fat cells[2,3]: The semicarbazide-sensitive amine oxidase (SSAO)[4] also known as amine oxidase copper containing 3[5] and identical to vascular adhesion protein (VAP-1)[6]. More precisely, it is hydrogen peroxide, one of the products of amine oxidation, and known from decades to stimulate glucose uptake in fat cells[7], that supports the insulin-mimetic actions of Bza in adipocytes, either in rodents[8] or in humans[9]. The *in vitro* insulin-like actions of Bza encompass activation of glucose uptake[10], induction of adipogenesis[11] , stimulation of lipogenesis [12], and inhibition of lipolysis. They occur even in the absence of insulin[8]. It was therefore of interest to investigate whether an oral treatment with Bza is capable of alleviating the impaired glucose handling of insulin-deficient, type 1 diabetic states.

Type 1 diabetes is characterized by a deficiency in insulin resulting from endocrine pancreas injury. To treat this disease, it is necessary to permanently normalize the altered blood glucose homeostasis. Since insulin is the major regulator of blood glucose levels, many therapeutic beneficial approaches have consisted in providing this pancreatic hormone, *via* repeated injections, or even by more sophisticated administration modes using biotechnologies, islet transplants or cell therapies[13]. Whatever the mode of supply, insulin overdose has to be avoided to prevent the risk of fatal hypoglycemia and to limit the onset of insulin resistance. Of note, various pharmacological agents or naturally occurring molecules can act as insulin-like factors on the glucose utilization by peripheral tissues[14]. In this view, testing the putative antihyperglycemic effect of Bza in type 1 diabetic rodents remains a preclinical step that deserves descriptive studies.

Alongside its capacity to oxidize Bza[1], fat tissue is not quantitatively but qualitatively of paramount importance in the regulation of glucose disposal. Adipose tissue uses glucose for accumulating lipid stores, and it also acts as an endocrine organ secreting a variety of adipokines with hyperglycemic or hypoglycemic properties, even in the absence of exogenous insulin[15]. The lack of adipose tissue

(lipoatrophy), such as that obtained in several genetically modified mice, is accompanied with altered glucose homeostasis[16,17]. Similarly, diabetic type 1 models, such as streptozotocin (STZ) diabetic rodents, with destroyed endocrine pancreas, exhibit reduced fat stores[18,19]. In humans, successful treatment of type 1 diabetes is concomitant with both restoration of normal glucose levels and adipose tissue recovery[20].

More importantly, diabetic phenotypes of diverse animal models have been ameliorated when white adipose tissue (WAT) or brown adipose tissue (BAT) has been reintroduced in these models, irrespective of the method used. Nowadays, it is suggested that adipose tissue contributes to the correction of type 1 diabetes, since hyperglycemia was lowered in diabetic mice treated by conditioned media from adipose-derived stem cells[21], and since mitigation of diabetes was observed in STZ diabetic mice receiving BAT transplantation[19]. To date, the beneficial effects of ingested Bza on glucose and lipid handling have been studied in obese rodents only[1,22]. These studies have suggested that enhanced fat deposition contribute to the insulin-like effects observed *in vivo*. Again, these considerations reinforced our interest in investigating the effects of Bza in a lipoatrophic model of type 1 diabetes.

The capacity of Bza to activate glucose transport in rat or mouse adipocytes is potentiated by the presence of vanadium[10,23], a widely recognized insulin-like agent[24,25]. Accordingly, it has been already demonstrated that *in vivo* treatments with a combination of amine oxidase substrates and vanadium exert antidiabetic effects in diverse diabetic rodents, including the STZ diabetic rats[10,26]. However, we demonstrated in recent studies that Bza[9] or catecholamines[27] are capable of activating glucose transport in human adipocytes, even in the absence of vanadium, and that the synergism vanadate/amine is much more weak in human adipocytes than in the murine ones. All these observations prompted us to examine for the first time the influence of prolonged oral administration of Bza alone-without any added vanadate—in a model of type 1 diabetes, which is nonobese and insulin-deficient; the STZ-induced diabetic mouse.

We investigated whether Bza alone was able, *via* oral consumption, to improve glucose handling in insulin-deficient STZ mice. The following results do not confirm our assumption, although they suggest that Bza action on glucose disposal requires a minimal amount of adipocytes prone to increase their glucose consumption when oxidizing this SSAO substrate.

MATERIALS AND METHODS

Chemicals

Benzylamine hydrochloride, STZ, bovine insulin, phenelzine, collagenase A, and most of the other reagents were from Sigma–Aldrich–Merck (Saint Quentin Fallavier, France). [^{3}H]-2-Deoxyglucose (2-DG) was from Perkin Elmer (Boston, MA, USA). The glucometers and consumables for follow-up of fed blood glucose were provided by Pr. Valet P. (Univ Toulouse, France), and used as previously described [28].

Insulin-deficient type 1 diabetic mice

Male Swiss mice obtained from Charles River Laboratories (L'arbresle, France) were housed at constant temperature (20–22°C) and with a 12-h light–dark cycle. At the age of 2 mo, they received an intraperitoneal injection of STZ (40 mg/kg) diluted in citrate buffer (0.05 mmol/L, pH 4.5) for four consecutive days, as described previously[21]. A week later, mice receiving only citrate buffer (nondiabetic) and treated mice exhibiting blood glucose ≥ 300 mg/100 mL (STZ diabetic) were subdivided into four groups of eight males, with either free access to water (control) or a 0.5% Bza solution as drinking liquid (Bza-drinking) for 24 d. To measure plasma insulin levels at the beginning of treatment, blood samples were withdrawn from tail vein then centrifuged and analyzed using Ultrasensitive insulin-ELISA kit (Mercodia, Uppsala, Sweden). All the mice had free access to food and water and were treated in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments)[29]. During this period, nonfasting blood glucose levels were determined every 3 d at 12:00 h (equivalent in the used circadian rhythm to 4 h after lights turned on) using an Accu-Check glucometer (Roche Diagnostics) on a blood drop withdrawn from the tail vein. Mice were killed after overnight fasting at the end of treatment and organs were collected and weighed.

Adipocyte preparations

Adipocyte preparations were obtained by collagenase digestion of WAT immediately after removal from the epididymal, intra-abdominal and inguinal anatomical locations. WAT was cut into small pieces, digested at 37°C by collagenase under agitation in Krebs–Ringer buffered at pH 7.5 with 15 mmol/L sodium bicarbonate, 10 mmol/L HEPES, supplemented with 3.5% of bovine serum albumin, as previously described[1]. Preparations of buoyant adipocytes were isolated from the digested WAT by filtration through nylon stockings and two gentle buffer washes, as described previously[10]. In our digestion process, approximately 1 g WAT was necessary to obtain sufficient functional adipocytes for the subsequent hexose uptake assays. When total amount of dissected WAT exceeded 1 g, excess

samples were snap-frozen at -80°C. This occurred for each of the normoglycemic mice but not for the lipoatrophic STZ-treated mice. In this case, pools of two mice were used to freeze approximately 200 mg WAT.

Glucose transport assays

The nonmetabolizable analog [^{3}H]-2-DG was the only source of hexose for the cell preparations during glucose transport assays. It was added at a final concentration of 0.1 mmol/L after 45 min incubation of the fat cell suspension with the tested agents, as previously described[10]. Pyruvate (2 mmol/L) was also present in the medium throughout the experiments for energy supply. Radioactive 2-DG (100 µL; approximately 1300000 dpm/vial) was added to 400 µL fat cell suspension, and hexose uptake assays were stopped 10 min later with 100 µL 100 µmol/L cytochalasin B. Cell suspensions (200 µL) were immediately transferred to plastic centrifugation microtubes prefilled with dinonyl-phthalate (density 0.98 g/mL), then subjected to a 30 s spin. The upper part of the tubes, containing radiolabelled hexose internalized in intact fat cells floating above the silicon layer was counted in scintillation vials, as described previously[10]. The extracellular [^{3}H]-2-DG present in the upper part of the tubes was determined in tubes receiving cytochalasin B prior to 2-DG. It averaged 1%–5% of the radioactivity found in control uptake, and was subtracted from all assays, as described previously[9].

Determination of benzylamine oxidation

Amine oxidase activity was determined at 37°C using [^{14}C]-Bza as substrate, in homogenates of thawed WAT samples, as previously described[10]. Isotopic dilution of [^{14}C]-Bza (final concentration: 0.1 mmol/L) was incubated for 30 min in 200 µL 200 mmol/L phosphate buffer with approximately 50 µg proteins, then the radiolabeled oxidation products were immediately extracted in toluene/ethyl acetate and counted as previously specified[9]. Results were expressed as nmol of deamination products/mg protein/min.

Statistical analysis

Results are presented as means ± SEM of (n) observations. All the statistical analyses for comparisons between parameters used ANOVA followed by *post hoc* Dunnett's multiple comparisons test, analyzed with Prism 6 for Mac OS X (GraphPad Software). Relative EC_{50} values were calculated by nonlinear regression.

RESULTS

Bza supplementation normalizes increased food and water consumption of STZ-induced diabetic mice without restoring body weight gain

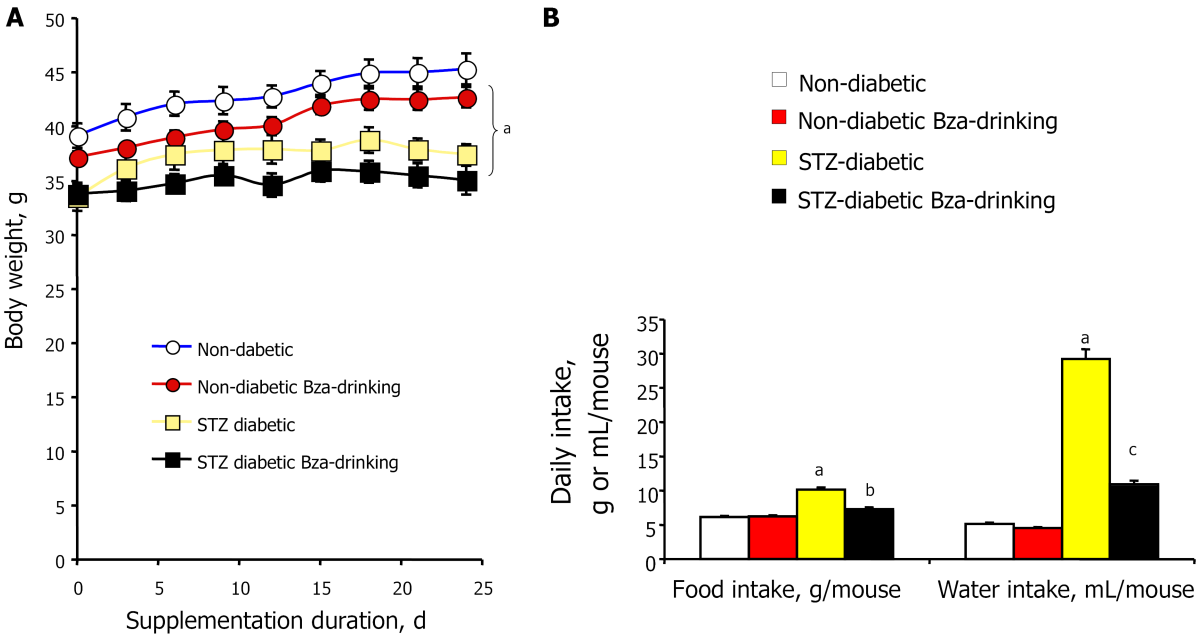
At the start of the experiment, the STZ-induced diabetic mice exhibited lower body weight when compared to age-matched normoglycemic mice (Figure 1). The body weight gain of the insulin-deficient mice was also limited during the treatment period and was not corrected by Bza supplementation. At the end of the experiment, the mean body weight of STZ mice remained lower than that of normoglycemic mice. Hence, Bza supplementation tended to limit body weight gain in both groups, but this trend did not reach significance (Figure 1A). No significant decrease in food consumption was found in the Bza-drinking normoglycemic mice. By contrast, the hyperphagic status of the STZ mice was alleviated by Bza supplementation (Figure 1B). A similar influence of Bza supplementation was found for water consumption. An almost normalization of the elevated daily water intake of STZ diabetic mice occurred in the group subjected to Bza drinking (Figure 1B).

Figure 1 also shows that the characteristic polydipsic feature that occurs in STZ-induced type 1 diabetes was of greater magnitude than the hyperphagy triggered by the noxious diabetogenic agent. The exaggerated liquid consumption of the diabetic group was increased by 5.7 times when compared to normoglycemic control while this increase only reached 1.7 times for food intake. The former defect was expected to traduce glycosuria[19,30], while the second likely corresponded to a lowered efficiency of the ingested carbohydrates that accompanies insulin deficiency[31].

In view of these alterations of food and water intake in STZ diabetic mice and their recovery after Bza drinking, the influence of Bza supplementation on blood glucose levels was examined in both fed and fasted conditions.

Influence of oral supplementation of Bza on blood glucose in nondiabetic and diabetic mice

Figure 2 shows the pattern of nonfasting glycemia during the treatment period for the four experimental groups. The unfasted blood glucose levels of the mice previously challenged with STZ were at least twice higher than those of the controls throughout the study (Figure 2A). Such strong hyperglycemia was mainly a consequence of the low circulating levels of insulin found at the start of treatment in the two groups of STZ diabetic mice (0.40 ± 0.04 and 0.38 ± 0.05 ng/mL) when compared to the nondiabetic mice (1.26 ± 0.14 and 1.35 ± 0.09 ng/mL, n = 8; P < 0.001). In the STZ diabetic mice, the blood glucose



DOI: 10.4239/wjd.v13.i9.752

Figure 1 Influence of Bza supplementation on body weight gain, food intake and water consumption in normoglycemic and STZ-induced diabetic mice. A: Body weight of diabetic (squares) and nondiabetic mice (circles) drinking water (open symbols) or 0.5% Bza (Bza-drinking, closed symbols). Mean ± SEM of n = 8 males in each group. Significant difference at: $^{a}P < 0.001$ between diabetic and nondiabetic mice, irrespective of the treatment; B: Average daily food intake and water intake. The mean daily consumption calculated throughout the treatment is expressed as g or mL/mouse, for each of the following groups: nondiabetic (white columns), nondiabetic Bza-drinking (red columns), STZ diabetic (yellow columns), STZ diabetic Bza-drinking (black columns). Each column is the mean ± SEM of at least 16 determinations. Different from nondiabetic rats at: $^{a}P < 0.01$. Different from respective control significant at: $^{b}P < 0.01$; $^{c}P < 0.001$. STZ: Streptozotocin; Bza: Benzylamine.

levels remained elevated in both Bza-drinking and water-drinking groups (Figure 2A). In the normoglycemic mice, the nonfasting blood glucose was superimposed in the control and Bza-drinking groups and remained below 200 mg/100 mL. Thus, blood glucose levels were not significantly influenced by repeated Bza consumption.

To avoid any alteration in body weight gain and in glucose handling, the mice were subjected to overnight fasting only once, at the end of experiment. Fasting blood levels were expectedly lower than nonfasting blood glucose (Figure 2B). Again, the fasting values were superimposable in Bza-drinking mice and their respective controls, while the fasting blood glucose of STZ diabetic mice was higher than that in nondiabetic groups (Figure 2B). Thus, Bza supplementation did not exhibit any hypoglycemic or antihyperglycemic action in this animal model of severe type 1 diabetes.

These findings contrasted with the capacity of Bza to delay the onset of diabetes in the genetically obese and diabetic *db*$^{-/-}$ mice[1]. Given the unexpected lack of efficiency of Bza consumption on glucose handling, it was poorly appropriate to delineate its putative mechanisms of action or to further examine other surrogate makers of diabetic state, as reported previously[1]. Instead, we verified whether the dose of Bza ingested was similar in the two diabetic models. Considering the daily liquid intake and the body mass of the STZ mice, it was calculated that these type 1 diabetic mice ingested 10850 ± 598 μmol/kg bw/d Bza throughout the treatment. This dose was similar to that used for Bza supplementation in young type 2 diabetic *db*$^{-/-}$ mice[1], which ranged between 9300 and 10 100 μmol/kg bw/d. However, another difference between type 2 (insulin-resistant) and type 1 (insulin-deficient) diabetic mouse models lies in the occurrence of excessive fat depots in the former and a clearly emaciated state in the latter. Therefore, attention was focused on WAT in the STZ mice and their controls.

Comparison of fat stores between normoglycemic and STZ-induced diabetic mice

Smaller mass of subcutaneous and visceral WAT was a typical feature of STZ-induced diabetic mice when compared to normoglycemic controls (Figure 3). In the STZ diabetic mice, the low mass of fat pads was not modified by Bza drinking, whatever their anatomical location. Similarly, the normal adiposity of the nondiabetic mice was not modified after oral Bza supplementation.

When the mass of the dissected fat depots was normalized as percentage of body weight, such adiposomatic index[22] was significantly lower in diabetic than in nondiabetic mice (1.2 ± 0.4% *vs* 3.7 ± 0.6%, $P < 0.001$). Again, Bza supplementation did not modify adiposomatic index: 1.3 ± 0.4% and 3.9 ± 0.4%, in Bza-drinking diabetic and nondiabetic groups, respectively.

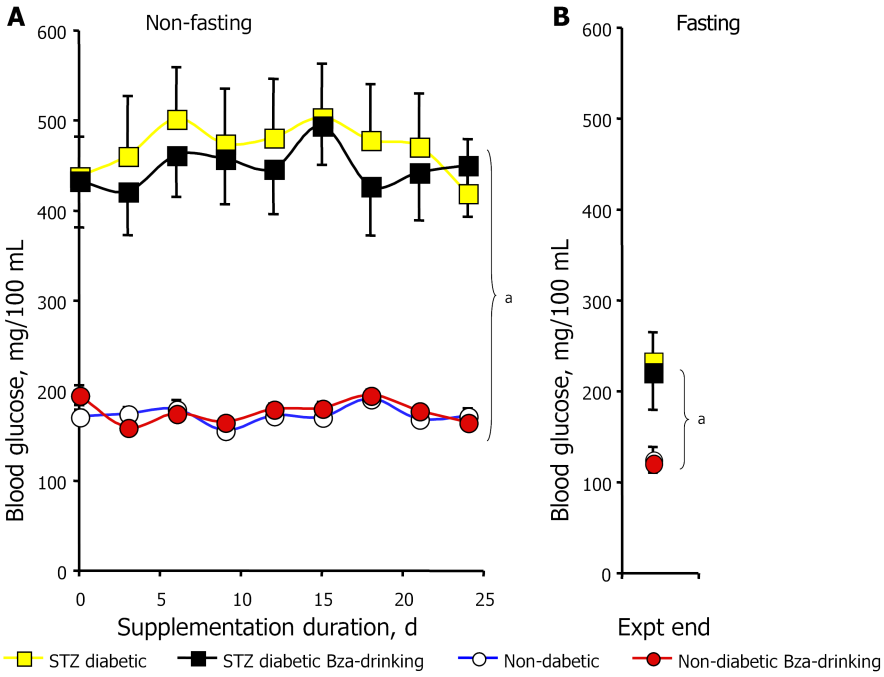


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Figure 2 Non-fasting and fasting blood glucose in normoglycemic and STZ-induced diabetic mice during Bza supplementation. A: Blood glucose measured every three days at 12:00 in diabetic (squares) and nondiabetic mice (circles) drinking water (open symbols) or Bza 0.5% (Bza-drinking, closed symbols) for 24 d; B: Overnight fasted blood glucose levels at the end of experiment for the same groups of mice. Mean ± SEM of n = 8 males in each group. Different from nondiabetic mice at: $^{a}P < 0.001$. No significant difference was found between Bza drinking and respective controls. STZ: Streptozotocin; Bza: Benzylamine; Expt end: The end of experiment.

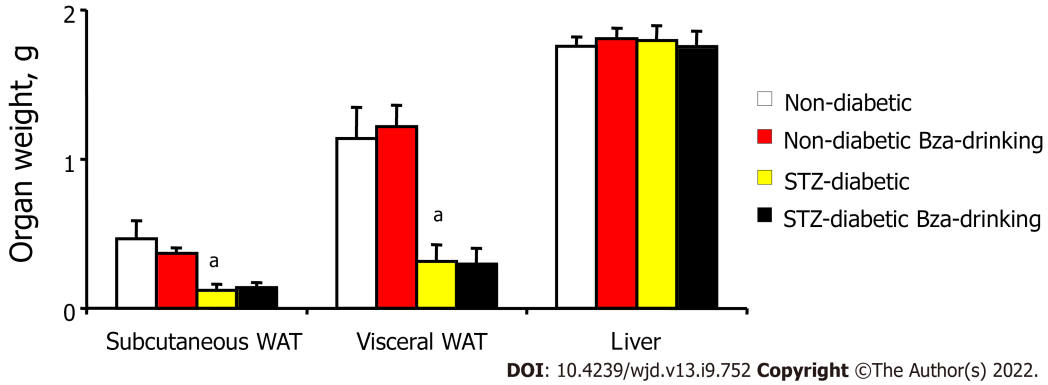

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Figure 3 Lack of influence of Bza supplementation on organ weight in normoglycemic and STZ-induced diabetic mice. Mean ± SEM of the wet weight of subcutaneous or visceral white adipose tissues, and of liver for eight males in each group. Different from nondiabetic mice at: $^{a}P < 0.001$. No significant difference was found between Bza-drinking and respective controls. STZ: Streptozotocin; Bza: Benzylamine; WAT: White adipose tissues.

In contrast, the weight of the liver was identical in the four experimental groups (Figure 3). However, when liver mass was expressed as ratio to body weight, the difference that appeared between diabetic and nondiabetic animals was opposite to that of the adiposomatic index. The liver represented 5.3 ± 0.2% of body mass in both STZ diabetic and STZ diabetic Bza-drinking mice (NS, n = 8). This proportion was smaller in nondiabetic mice (4.2 ± 0.1%, $P < 0.001$), even after Bza drinking (4.5 ± 0.2%).

These observations indicated that the STZ-induced diabetic mice did not normalize their reduced fat deposition and body weight gain after Bza supplementation, in spite of partial recovery of their altered food intake. Moreover, Bza supplementation was not efficient in normalizing the altered blood glucose control or relative hepatomegaly of the STZ mice, although limiting polydipsia. We have previously proposed that Bza oxidation occurring in the hypertrophied WAT of obese and diabetic $db^{-/-}$ mice supports its insulin-like *in vitro* effects by facilitating glucose utilization in adipocytes and contributes to its antihyperglycemic action[1]. Therefore, such *in vitro* effects were examined.

Effects of insulin and Bza on glucose transport in mouse adipocytes

Unfortunately, the WAT atrophy of the STZ diabetic mice did not allow the preparation of sufficient biological material for exploring the activation of 2-DG uptake in functional adipocytes from diabetic and Bza-drinking diabetic mice. There was only a pool of around 400 mg of WAT dissected from different anatomical locations in each STZ mouse, while 1–2 g was removed from each nondiabetic mouse. Consequently, sufficient adipocytes could be isolated from the latter samples only, and the subsequent hexose uptake assays were performed with adipocyte preparations that contained 18.0 ± 2.8 and 19.0 ± 2.5 mg lipid/400 µL in normoglycemic Bza-drinking and control mice, respectively. Thus, Figure 4A shows insulin stimulation of 2-DG uptake in nondiabetic mice only. As expected, insulin dose-dependently activated hexose uptake in adipocytes from control mice, and a tendency to improve insulin maximal effect was detected in Bza-drinking mice. EC_{50} values of insulin were 0.4 and 2.3 nmol/L for Bza-drinking and control mice, respectively, without showing a significant difference between them. Figure 4B indicates that 0.1 mmol/L benzylamine was capable of reproducing one-third of the maximal insulin stimulation, in a manner that was blunted by the amine oxidase inhibitor phenelzine, which was inactive on basal or insulin-stimulated hexose uptake. The amine-oxidase-dependent insulin-like effect of 0.1 mmol/L Bza was similar in control and Bza-drinking nondiabetic mice. There was no influence of oral Bza supplementation on the capacity of phenelzine to inhibit *in vitro* the insulin-like action of the amine (Figure 4B).

Oxidation of Bza in thawed preparations of adipose tissues

Amine oxidase activity was determined in homogenates from thawed WAT samples by measuring their capacity to oxidize 0.1 mmol/L [^{14}C]-Bza. When expressed as nmol amine oxidized/mg protein/min, the activity was limited in WAT from STZ diabetic mice compared to normoglycemic ones, whether in the control or Bza-drinking groups (Figure 5). The reduced amount of WAT and its limited amine oxidase activity did not argue for a strong contribution of fat stores to the biotransformation of the Bza ingested by STZ diabetic mice.

DISCUSSION

At the first glance, the lack of antihyperglycemic effect of Bza drinking described here in STZ diabetic mice contrasts with its antidiabetic action observed in obese and diabetic *db*$^{-/-}$ mice[1]. As discussed below, all these findings converge to propose that the difference in Bza-drinking efficiency between the models of type 1 and type 2 diabetes is not related to insulin deficiency *versus* resistance, but rather to a difference in adiposity between the murine models.

Alongside bearing dramatically larger fat depots than their lean counterparts, the obese and diabetic *db*$^{-/-}$ mice also possess higher levels of SSAO activity in their fat cells[1,32]. Thus, the antihyperglycemic effect of oral Bza reported for *db*$^{-/-}$ mice, and not for their lean littermates, could be related to the elevated amine oxidase activity found in the hypertrophied WAT of obese and diabetic animals[1]. In contrast, STZ diabetic rats exhibit lower monoamine oxidase (MAO) and SSAO activities in WAT than their normoglycemic controls[18]. The lack of antihyperglycemic effect of Bza supplementation in STZ mice reported here resembles the weak antidiabetic effect of prolonged administration of tyramine in STZ rats[18]. Tyramine, which is a substrate of both MAO and SSAO, can limit the hyperglycemic responses to a glucose load during a glucose tolerance test but cannot normalize the elevated fasting blood levels of these insulin-deficient rats. Tyramine or Bza can lower the elevated blood glucose of STZ-induced diabetic rats only when combined with vanadium[10,18,33].

Particular attention has been paid to studying the potential antidiabetic effects of amines alone since the synergism between vanadium and biogenic amines on the activation of glucose transport does not work well in human adipocytes[9,27]. Moreover, the potential antidiabetic use of vanadium derivatives is still limited by toxicological aspects. Several observations suggest that the beneficial effects of dietary amines on glucose handling in diabetic rodents (even when not combined with vanadium) rely upon the amount of SSAO present in WAT. The supplementation of drinking water with 0.4% methylamine (another SSAO substrate) has been reported to increase epididymal WAT mass and to improve glucose tolerance in transgenic mice overexpressing a human form of SSAO/VAP-1, while it is inefficient in nontransgenic mice[34]. Oral Bza also improves glucose handling in high-fat diet fed mice, characterized by increased adiposity[22]. Here, we suppose that it is the lipoatrophy of STZ diabetic mice (and not their lack of insulin) that prevented the occurrence of an antihyperglycemic action of Bza.

The sole beneficial effect of Bza drinking seen in the STZ diabetic mice was an almost total recovery of their characteristic hyperphagic and polydipsic behavior[31]. It could be supposed that urinary glucose leak of STZ mice was partially rescued by Bza drinking. Unfortunately, individual metabolic cages were not available for this study and we could not determine daily urine emission or glucosuria. However, water intake reduction occurred without correction of hyperglycemia. This indicated that renal glucose leak, if any, was not sufficiently rescued by Bza drinking to influence the overall glucose homeostasis, while this was the case for *db*$^{-/-}$ mice[1]. Food intake was also reduced in Bza-drinking STZ diabetic mice, but without notable decrease in body weight gain. Thus, food efficiency was increased by Bza drinking.

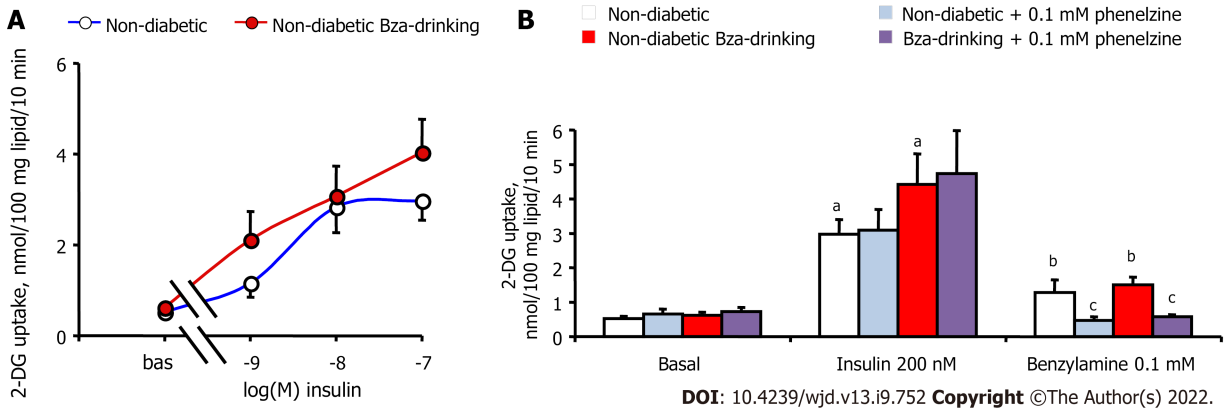

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Figure 4 Direct stimulation by insulin and Bza of hexose uptake in adipocytes: lack of influence of Bza supplementation and *in vitro* inhibition by phenelzine. A: Radiolabeled 2-deoxyglucose (2-DG) uptake was determined in basal condition or in response to increasing doses of insulin in adipocytes from nondiabetic mice of the water-drinking (open circles) or Bza-drinking (red circles) group, while it could not be determined in diabetic mice due to the scarcity of adipocytes isolated from their emaciated fat depots, in both control and Bza-drinking groups. Mean ± SEM of eight adipocyte preparations. B: 2-DG uptake was determined after 45 min incubation without (basal) or with 200 nmol/L insulin and 0.1 mmol/L Bza. The stimulated hexose uptake was significantly different from basal at: $^{a}P < 0.001$; $^{b}P < 0.01$. Phenelzine was added at 0.1 mmol/L in the incubation medium of adipocytes from control nondiabetic mice (blue columns) or from Bza-drinking nondiabetic mice (purple columns). Phenelzine inhibited significantly Bza-induced hexose uptake at: $^{c}P < 0.01$. STZ: Streptozotocin; Bza: Benzylamine; WAT: White adipose tissues.

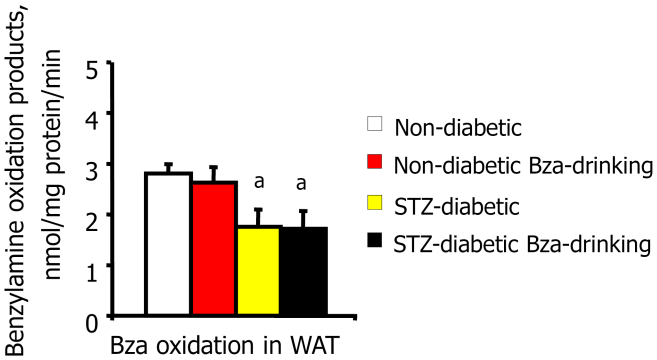

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Figure 5 Bza oxidation in adipose tissue of normoglycemic and STZ-induced diabetic mice: lack of influence of dietary Bza consumption. Radiolabeled Bza was present at 0.1 mmol/L during 30 min incubation at 37°C with WAT homogenates from nondiabetic (white columns), nondiabetic Bza-drinking (red columns), STZ diabetic (yellow columns), STZ diabetic Bza-drinking (black columns). Mean ± SEM of eight determinations for nondiabetic mice and four determinations for lipoatrophic STZ diabetic mice. Different from nondiabetic at: $^{a}P < 0.02$. No significant influence of Bza-treatment was detected. STZ: Streptozotocin; Bza: Benzylamine; WAT: White adipose tissues.

However, we cannot propose any underlying mechanism for this effect.

Indeed, it cannot be excluded that mechanisms other than oxidation by amine oxidases might be involved in the *in vivo* effect of Bza on food and water intake. Raimondi and coworkers have reported that Bza, like methylamine, rapidly induces hypophagia in mice *via* a modulation of neuronal channels, which is reinforced by SSAO inhibition[35,36]. This suggests that adipose SSAO is likely not the sole target of ingested Bza. Regarding activation of glucose uptake in adipocytes, the effect of Bza is impaired when its oxidation by SSAO is blocked. Surprisingly, the opposite occurred regarding its central effects on food and water intake. When Bza degradation by SSAO is blocked, its half-life is increased and its capacity to modulate the neuronal channels depicted by the group of Raimondi is improved[35,36]. Since there is practically no WAT in the STZ-diabetic mice, and since they have little adipose SSAO, we propose that the limitation of hyperphagia and polydipsia observed in these animals is likely due to a central effect distinct from oxidation by peripheral tissues.

Although the liver is another of the organs reached by ingested Bza, it is not a major site for its biotransformation or detoxification because Bza is metabolized to only a small extent by hepatic subcellular fractions, as observed by Mutlib *et al* [37]. By contrast, these authors reported that, when orally given to rats, Bza undergoes oxidative deamination and generates benzaldehyde, then hippuric acid, which is the major metabolite. These authors also observed that Bza was fairly stable in rat plasma despite of the presence of a soluble form of SSAO. Although circulating SSAO activity is known to

increase with diabetes[18,38-40], it is low when compared to the levels of SSAO found in WAT[1]. A putative mediation of the amine effects *via* modulation of insulin secretion can be ruled out because, in another model of insulin-deficient diabetes, the alloxan-injected rat, oral administration of tyramine reduced the hyperglycemia by 35%–43% in a manner that was more dependent on insulin-like than on insulin-releasing actions[41].

A limitation of the study was that insulin plasma levels were not determined throughout the treatment since such measurements were performed only at the beginning. However, since circulating insulin was dramatically decreased by STZ challenge, and since the overt hyperglycemia was not corrected by Bza drinking, it was hypothesized that pancreatic injury was not recovered. The hyperinsulinemic levels of the insulin-resistant $db^{-/-}$ mice remained unchanged after Bza supplementation[1]. Similarly, no change in plasma insulin was found in the $db^{+/+}$ lean control after Bza drinking. Nonetheless, it has been reported that methylamine (another SSAO substrate) limits the insulin degradation by adipocytes[42]. If one supposes that increasing the ability of insulin to stimulate glucose transport is one of the mechanisms involved in the antidiabetic effect of Bza, this can explain why Bza was active in insulin-resistant but not in insulin-deficient diabetes models. Such a paradigm of insulin-sensitizer capacity might provide an alternative to our interpretations based on the necessary abundance of SSAO and WAT to support peripheral glucose disposal. However, it requires to be demonstrated by further investigations, while we report in the current study that Bza alone activated 2-DG uptake in adipocytes, being therefore able to act as an insulin mimicker even in the absence of insulin.

Whether the *in vitro* SSAO-mediated insulin-like effect of Bza is solely responsible for the antihyperglycemic effect of Bza drinking is far from being demonstrated here. However, this assumption agrees with the conclusions of independent studies showing that treatment of diabetic rodents with SSAO inhibitors prevents diabetic complications but is not antihyperglycemic at all[43-45]. All these observations bring evidence that adipose cells are predominantly involved in Bza oxidation, as a consequence of their high SSAO expression[3], although they do not rule out other concomitant mechanisms.

We designed the current study to achieve a similar daily amount of Bza ingested by the STZ diabetic mice to that ingested by the obese and type 2 diabetic $db^{-/-}$ mice[1]. The results showed that such an objective was reached. However, similar amine intake did not result in a similar beneficial influence on glucose disposal in the two models. In the STZ diabetic mice, the lipoatrophy and lower richness of WAT in amine oxidase activity gave less probability for an adipocyte-dependent metabolism of the ingested amine and subsequent insulin-like actions. Another apparent weakness of the present study was that the nondiabetic Swiss mice did not ingest the same daily amount of Bza than those subjected to the STZ diabetogenic challenge. Our experiments showed that the polydipsia of the STZ diabetic mice was early rescued, after the first week of Bza supplementation. They also showed that, among the Bza-drinking groups, the accumulated fluid intake of the STZ diabetic mice was about twice that of the normoglycemic mice. It could be easily justified *post hoc* that, considering the initial polydipsia of diabetic mice, it would have been preferable to double the Bza concentration in the solution given to the Bza-drinking nondiabetic group. Hence, it cannot be excluded that such a high dose of Bza would have reduced liquid consumption in the nondiabetic mice also. By assumption, such an adverse effect on liquid consumption remains unlikely since, as with other organic amines, Bza has a taste varying from almond to fish waste[46], which is not supposed to be repellent for rodents. In reality, achieving exactly the same oral dose of Bza for diabetic and nondiabetic animals would have required weekly pair-adjustments, which are difficult to achieve, and would not have yielded more information about the mechanisms of action. The unchanged lipoatrophy, together with the early recovery of polydipsia in the Bza-drinking group, converge to indicate that the antipolydipsic effect of the amine is mediated by a central effect, distinct from that observed in adipocytes.

The *in vitro* insulin-like effect of submillimolar dose of Bza on glucose transport in adipocytes, and its blockade by phenelzine, reinforced our hypothesis of enhancement of peripheral glucose disposal, although it could not be evidenced in lipoatrophic Bza-drinking STZ mice. Phenelzine, which is a combined MAO and SSAO inhibitor, was used because both MAO and SSAO substrates mimic insulin-like effects in adipocytes[33]. It blocked Bza-stimulated hexose uptake, but not basal or insulin-stimulated hexose uptake. No resistance to the selective blockade by phenelzine appeared in the fat cells from Bza-drinking nondiabetic mice, indicating that continuous supplementation with the substrate did not dramatically downregulate the amine oxidase activities. These hexose uptake assays, which could be performed on nondiabetic mice only, confirmed that, even in the absence of insulin, Bza oxidation activates hexose uptake in adipocytes from Swiss white mice as well as in other rodents[43]. According to the literature, the increase of glucose transport by SSAO activation is limited to adipocytes, and only rare reports have extended this hydrogen-peroxide-dependent insulin-like action to other cell types[47]. Unfortunately, the insufficient number of adipocytes isolated from the atrophied WAT of STZ mice hampered the verification of glucose transport responsiveness to insulin and Bza in the type 1 diabetic state. Even if such insulin mimicry also occurred in adipocytes from insulin-deficient mice, it was too limited to modify the glucose handling, when considering the low mass of WAT, as attested by the significantly lower adiposomatic index found in STZ-treated mice. The limited oxidative metabolism of Bza found in WAT of STZ mice was likely unable to contribute to a replenishment of the atrophied fat

depots *via* the increase of glucose utilization demonstrated in adipocytes of the normoglycemic controls.

Being poorly biotransformed by the limited fat stores of STZ diabetic mice, the ingested Bza could not increase glucose entry in adipocytes and thereby did not contribute to glucose disposal. We presume that such a lack of Bza action explains how its consumption did not decrease elevated blood glucose. Such inefficiency does not preclude future improvements of the antidiabetic therapeutic applications of other amine substrates. However, our findings limit the relevance of Bza consumption to alleviate the complications of type 1 diabetes, especially when accompanied with lipoatrophy. Nevertheless, Bza and its derivatives remain potential antihyperglycemic agents since a recent integrated network pharmacology analysis has revealed that Bza derivatives contribute to the anti-insulin resistance effects of *Moringa oleifera*[48], one of the most potent antidiabetic medicinal plants[30,49,50].

CONCLUSION

Although Bza drinking is devoid of beneficial *in vivo* effects on the type 1 diabetes at doses that limit the onset of type 2 diabetes in genetically obese $db^{-/-}$ mice, the present findings reinforce the hypothesis that oxidation of Bza at the level of adipocytes contributes to peripheral glucose uptake and improves glucose homeostasis. When no sufficient WAT is present (in STZ diabetic mice), the antihyperglycemic effect of Bza is hampered. In contrast, when Bza can be readily oxidized in WAT, it improves glucose tolerance at the expense of an enlargement of fat stores (in $db^{-/-}$ mice). The *in vitro* experiments confirmed the capacity of submillimolar doses of Bza to activate glucose transport in adipocytes. They also show that such SSAO-dependent insulin mimicry is not altered by chronic administration of the substrate.

ARTICLE HIGHLIGHTS

Research background

Oral administration of benzylamine (Bza) exerts antihyperglycemic effects in obese and diabetic rodent models. This effect has been proposed to depend on the insulin-like action of Bza in adipose cells. The amine oxidation catalyzed by amine oxidases abundantly present in adipocytes generates hydrogen peroxide, which activates glucose transport.

Research motivation

To extrapolate the potential antihyperglycemic properties of Bza found in obese and diabetic models to the treatment of insulin-deficient type 1 diabetic states. Bza administration might facilitate glucose utilization to increase lipogenic and adipogenic activities in the adipose tissue and thereby improve glucose disposal even in the absence of insulin.

Research objectives

To evaluate the impact of Bza supplementation on hyperglycemia, polydipsia and hyperphagia in type 1 diabetic mouse, and to demonstrate that Bza metabolism by adipose tissue supports these antidiabetic effects.

Research methods

Bza solution (5 g/L, Bza-drinking) replaced drinking water in streptozotocin (STZ)-induced, insulin-deficient diabetic mice. Similar comparison between control and Bza-drinking groups was performed in normoglycemic mice. Nonfasting blood glucose, water and food intake were periodically recorded in the four groups. Adiposity was determined at the end of a 24-d treatment. Glucose transport in freshly isolated adipocytes was assessed *ex vivo* by determining the uptake of the nonmetabolizable radiolabeled 2-deoxyglucose.

Research results

Chronic Bza intake did not normalize hyperglycemia in STZ diabetic mice, despite it alleviating excessive water and food consumption. Bza intake had no effect on the limited body weight of the STZ diabetic mice and could not restore their dramatically reduced adipose tissue mass. In normoglycemic mice, the Bza-drinking group did not show altered body weight, or food or water consumption. However, when directly given *in vitro* to adipocytes isolated from nondiabetic mice, Bza was efficient in activating glucose uptake in both control and Bza-drinking groups.

Research conclusions

The capacity of Bza supplementation to reduce hyperglycemia, previously reported in obese and diabetic rodents, was not detectable in the emaciated and insulin-deficient STZ diabetic mice. However, the capacity of Bza to activate glucose transport in adipocytes was confirmed in nonobese, nondiabetic

mice. It is likely that the adipose tissue atrophy induced by STZ challenge hampered the lipogenic and adipogenic action of Bza in this severe model of lipoatrophic, insulin-deficient diabetes.

Research perspectives

The current findings and their interpretations considerably limit the field of applications of oral Bza since this molecule did not work as an antidiabetic agent in rodents with reduced adiposity, as it is the case in type 1 STZ diabetic and lipoatrophic mice. Nevertheless, since SSAO substrates exhibit a direct action on glucose handling by fat cells, they still have potential interest for therapeutic use to combat other diabetic states.

ACKNOWLEDGEMENTS

We thank Thomas Cadoudal (I2MC, Toulouse, France) for managing STZ treatment and subsequent hyperglycemic and insulin deficiency screening. The authors also thank Ana Gomez-Ruiz and Sophie Fonvieille (I2MC, Toulouse, France) for help in the follow-up, and for disposable plastic supply, respectively. Special thanks to Philippe Valet and Anne Bouloumié (Univ. Toulouse) for sharing their invaluable knowledge about obese and diabetic rodents.

FOOTNOTES

Author contributions: Carpéné C designed the studies, isolated cells for *in vitro* experiments, reviewed the literature, designed the figures, wrote and revised the manuscript; Mercader Barceló J performed animal treatments, non-invasive and *ex vivo* explorations, Stiliyanov Atanasov K was involved in data mining, Les F contributed to statistical analysis, literature review and revised the manuscript.

Institutional review board statement: The study was approved by the I2MC Institutional Review Board: Institut des maladies métaboliques et cadiovasculaires (http://www.i2mc.inserm.fr/accueil).

Institutional animal care and use committee statement: Mice were housed and manipulated according to the INSERM guidelines and European Directive 2010/63/UE by competent and expert technicians or researchers in animal care facilities with agreement number A 31 555 011. The experimental protocol was approved by the local ethical committee CREFRE.

Conflict-of-interest statement: The authors declare no competing financial interests.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guideline.

Country/Territory of origin: France

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S-Editor: Chang KL
L-Editor: Kerr C
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 765-775

DOI: 10.4239/wjd.v13.i9.765

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Role of insulin in pancreatic microcirculatory oxygen profile and bioenergetics

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Diane A, Qatar; Jovandaric M, Serbia; Trujillo X, Mexico

Received: April 29, 2022
Peer-review started: April 29, 2022
First decision: May 29, 2022
Revised: June 9, 2022
Accepted: August 25, 2022
Article in press: August 25, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

The pancreatic islet microcirculation adapts its metabolism to cope with limited oxygen availability and nutrient delivery. In diabetes, the balance between oxygen delivery and consumption is impaired. Insulin has been proven to exert complex actions promoting the maintenance of homeostasis of the pancreas under glucotoxicity.

AIM

To test the hypothesis that insulin administration can improve the integrated pancreatic microcirculatory oxygen profile and bioenergetics.

METHODS

The pancreatic microcirculatory partial oxygen pressure (PO_2), relative hemoglobin (rHb) and hemoglobin oxygen saturation (SO_2) were evaluated in nondiabetic, type 1 diabetes mellitus (T1DM), and insulin-treated mice. A three-dimensional framework was generated to visualize the microcirculatory oxygen profile. Ultrastructural changes in the microvasculature were examined using transmission electron microscopy. An Extracellular Flux Analyzer was used to detect the real-time changes in bioenergetics by measuring the oxygen consumption rate and extracellular acidification rate in islet microvascular endothelial cells (IMECs).

RESULTS

Significantly lower PO_2, rHb, and SO_2 values were observed in T1DM mice than in

nondiabetic controls. Insulin administration ameliorated the streptozotocin-induced decreases in these microcirculatory oxygen parameters and improved the mitochondrial ultrastructural abnormalities in IMECs. Bioenergetic profiling revealed that the IMECs did not have spare respiratory capacity. Insulin-treated IMECs exhibited significantly greater basal respiration than glucotoxicity-exposed IMECs ($P < 0.05$). An energy map revealed increased energetic metabolism in insulin-treated IMECs, with significantly increased ATP production, non-mitochondrial respiration, and oxidative metabolism (all $P < 0.05$). Significant negative correlations were revealed between microcirculatory SO_2 and bioenergetic parameters.

CONCLUSION
Glucotoxicity deteriorates the integrated pancreatic microcirculatory oxygen profile and bioenergetics, but this deterioration can be reversed by insulin administration.

Key Words: Diabetes mellitus; Glucotoxicity; Endothelial cells; Microcirculation; Mitochondria; Bioenergetics

Core Tip: The pancreatic islet microvasculature adapts its metabolism to cope with limited oxygen availability and nutrient delivery. Insulin has been proven to exert complex actions promoting the maintenance of homeostasis under glucotoxicity. Our findings demonstrate that insulin ameliorates the suppression of the integrated microcirculatory oxygen profile in type 1 diabetes mellitus mice and improves mitochondrial ultrastructural abnormalities in islet microvascular endothelial cells (IMECs). Additionally, insulin administration restores glucotoxicity-induced microcirculatory failure by increasing the mitochondrial basal respiration and glycolytic capacity of IMECs.

Citation: Li BW, Li Y, Zhang X, Fu SJ, Wang B, Zhang XY, Liu XT, Wang Q, Li AL, Liu MM. Role of insulin in pancreatic microcirculatory oxygen profile and bioenergetics. *World J Diabetes* 2022; 13(9): 765-775
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/765.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.765

INTRODUCTION

The concept of pancreatic islet microcirculation, which is currently under the spotlight[1,2], is responsible for coupling metabolic demands with glucose distribution and oxygen delivery in a manner involving microvascular endothelium-dependent vasodilation. Emerging evidence, including ours, indicates that the integrated pancreatic microcirculation in islets is necessary to maintain endocrine function and is involved in the pathogenesis of diabetes, partially through impairment of microcirculatory blood perfusion[3,4].

As part of a highly specialized microvascular system[5], pancreatic islets are richly vascularized and have 5-10-fold higher blood flow than the exocrine pancreas[6]. Pancreatic islet microvascular endothelial cells (IMECs) are therefore responsible for maintaining substance exchange and contribute to the dynamic regulation of glucose metabolism. Malfunction of IMECs is not only the culprit of deteriorated pancreatic islet microvascular blood perfusion and oxygen supply but also a victim of imbalanced energetic homeostasis.

Metabolic abnormalities in glucose are generally related to alterations in energy metabolism, especially at the onset of diabetes. The main organelle of IMECs responsible for energetic homeostasis is the mitochondrion, which plays a critical role in IMEC survival and death by regulating ATP synthesis through glucose metabolism, ROS generation, and apoptosis[7,8]. Furthermore, the metabolic profile of IMECs links the microcirculatory phenomena to the occurrence of pathological phenotypes. It is therefore important and rational to investigate the metabolic states of IMECs to clarify the microcirculatory pathological changes that occur under glucotoxicity.

Several reports have suggested the involvement of microcirculatory endothelial dysfunction in diabetes. However, knowledge surrounding the bioenergetics of IMECs related to insufficient microcirculatory oxygen is rather limited. We have established a new microcirculatory monitoring approach that integrates pancreatic microcirculatory partial oxygen pressure (PO_2), relative hemoglobin (rHb) and hemoglobin oxygen saturation (SO_2) using a multimodal device[9]. Thus, the purpose of this study was to describe the integrated pancreatic microcirculatory oxygen under glucotoxicity and to determine the impact of insulin on the microcirculatory oxygen and bioenergetic profile of IMECs.

MATERIALS AND METHODS

Animals

BALB/c mice were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS). The mice were bred and housed at 22 ± 1 °C with 55%-65% humidity under a 12 h:12 h light:dark cycle. The mice were randomly divided into three groups, including a type 1 diabetes mellitus (T1DM) model group, an insulin-treated group and a nondiabetic control group (all n = 3). T1DM was established by intraperitoneal administration of streptozotocin (STZ, 40 mg/kg) into the mice for five consecutive days. A level of blood glucose higher than 200 mg/dL was considered to indicate diabetes. Insulin was subcutaneously injected (1.5 IU/day) into the mice in the insulin-treated group to maintain the blood glucose within the normal range[10]. The animal experiments in this study were permitted by the Laboratory Animal Welfare and Ethics Committee, Institute of Microcirculation, CAMS (China).

Measurements of the microcirculatory oxygen profile

To assess pancreatic microcirculatory oxygen, we employed a multimodal auxiliary microcirculatory monitoring system established with a fiber-optic oxygen sensor (Precision Sensing, Regensburg, Germany) and an Oxygen to See device (LEA Medizintechnik, Giessen, Germany) to determine the SO_2, rHb, and PO_2. After anesthesia, the pancreas was gently exposed by a median abdominal incision, and the microcirculatory oxygen profile, including SO_2, rHb, and PO_2, was subsequently captured. These parameters were measured at three random sites of the pancreas in each mouse.

Establishment of the three-dimensional framework of the microcirculatory oxygen profile

Python (ver 3.7.4) and Apache ECharts (ver 4.2.0-rc.2) were used to generate a three-dimensional framework to visualize the pancreatic microcirculatory oxygen profile. In the 3-D framework, the X-, Y-, and Z-axes represented the time course, microcirculatory oxygen variables, and calculated values of the microcirculatory oxygen profile, respectively. The outliers were adjusted by the box plot algorithm. Additionally, the least common multiple algorithm was used to adjust the time granularity. Min-max normalization was conducted to transform the dimensions of multiple parameters.

Video recording of the microcirculatory oxygen profile

ScreenToGif (version 2.19.3) was used to capture the dynamic 3-D framework. Each video was recorded in an MPEG4 file. The bitrate was 2000 Kbps with a 1920 × 1080 resolution ratio.

Transmission electron microscopy

Ultrastructural changes in the pancreatic islet microvasculature were examined using transmission electron microscopy (TEM). Fresh pancreatic tissue was fixed in 3% glutaraldehyde and 1% osmic acid and then passed through a graded series of dehydration and embedding solutions. Ultrathin sections (70 nm) were made after resin polymerization and uranyl acetate/Lead citrate staining. The samples were examined using a JEM-1400Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan). The mitochondrial ultrastructure of IMECs was assessed.

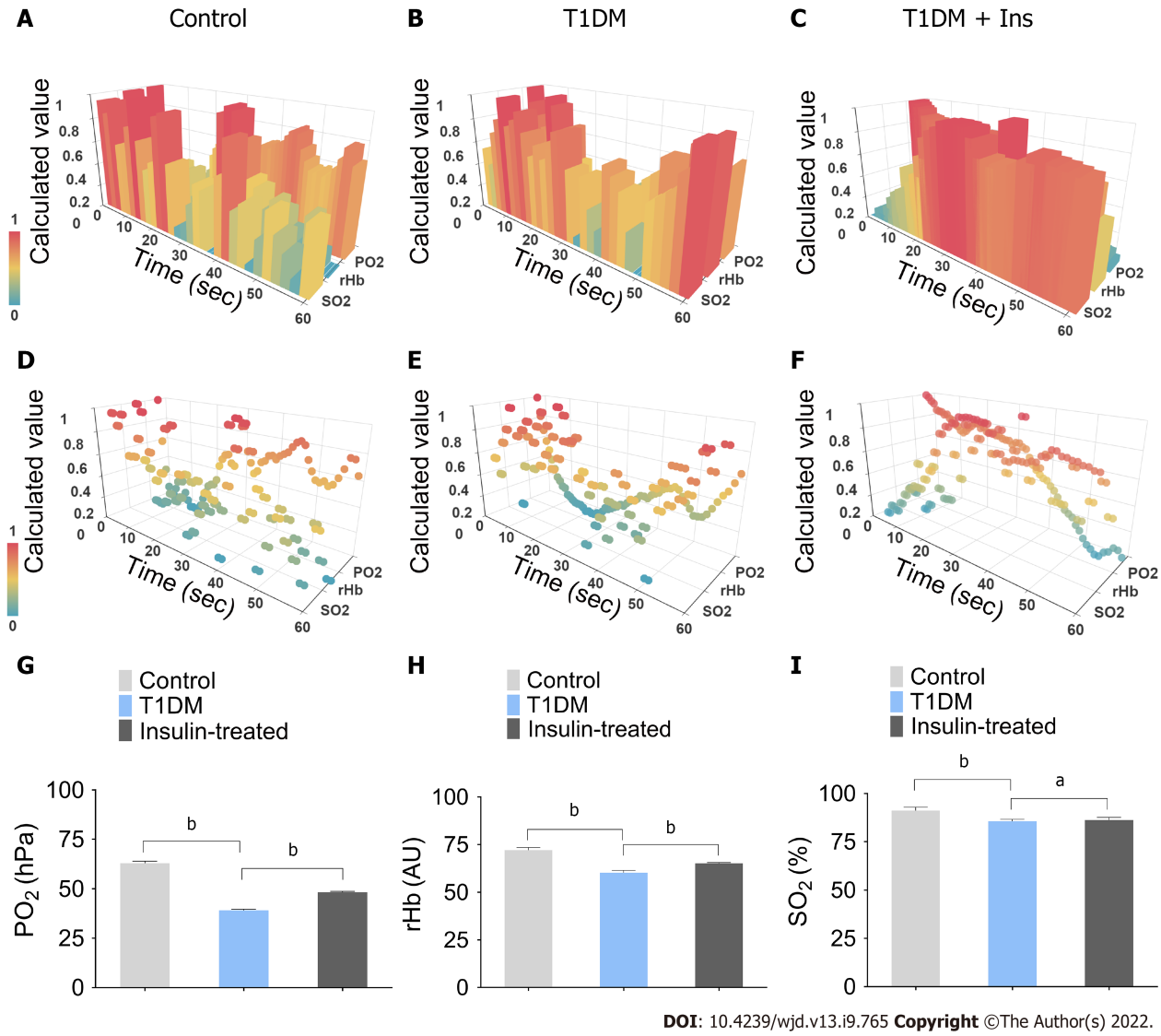
Cell culture

The IMECs were purchased from ATCC (MS1, Manassas, VA, United States) and routinely cultured in DMEM supplemented with 10% FBS, 5.6 mmol/L glucose, 2% HEPES and 100 U of penicillin–streptomycin (Gibco, Carlsbad, CA, United States). After IMECs grew to confluence, the cells were divided into three groups, the control, glucotoxicity-exposed (HG, 25 mmol/L glucose), and insulin-treated (HG + Ins, 25 mmol/L glucose plus 10^{-8} mol/L insulin[10]) groups, and were treated for 24 h (n = 4 each group).

Bioenergetics assay

To investigate the effects of the high concentration of glucose with or without insulin on the bioenergetics of IMECs, an Extracellular Flux Analyzer (XFe24, Seahorse Bioscience, Billerica, MA, United States) was used to detect the real-time changes in energy pathways by directly probing the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Briefly, IMECs were seeded in XFe24 culture plates at 1×10^4 cells per well. The cells were treated according to the abovementioned grouping for 24 h in DMEM with 0.5% FBS. The medium was subsequently replaced by Seahorse XF assay medium, and the cells were incubated without CO_2 for 1 h until detection.

For the mitochondrial stress test, mitochondrial function was monitored along with the sequential addition of oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and rotenone/antimycin A (all 0.5 μM). Multiple respiratory indexes, including baseline metabolic functions (basal respiration, proton leak, ATP-linked respiration, non-mitochondrial respiration, and oxidative metabolism) and metabolic stress responses (maximum respiratory capacity [MRC] and spare respiratory capacity [SRC]), were analyzed and compared. In addition, an ECAR value was probed to



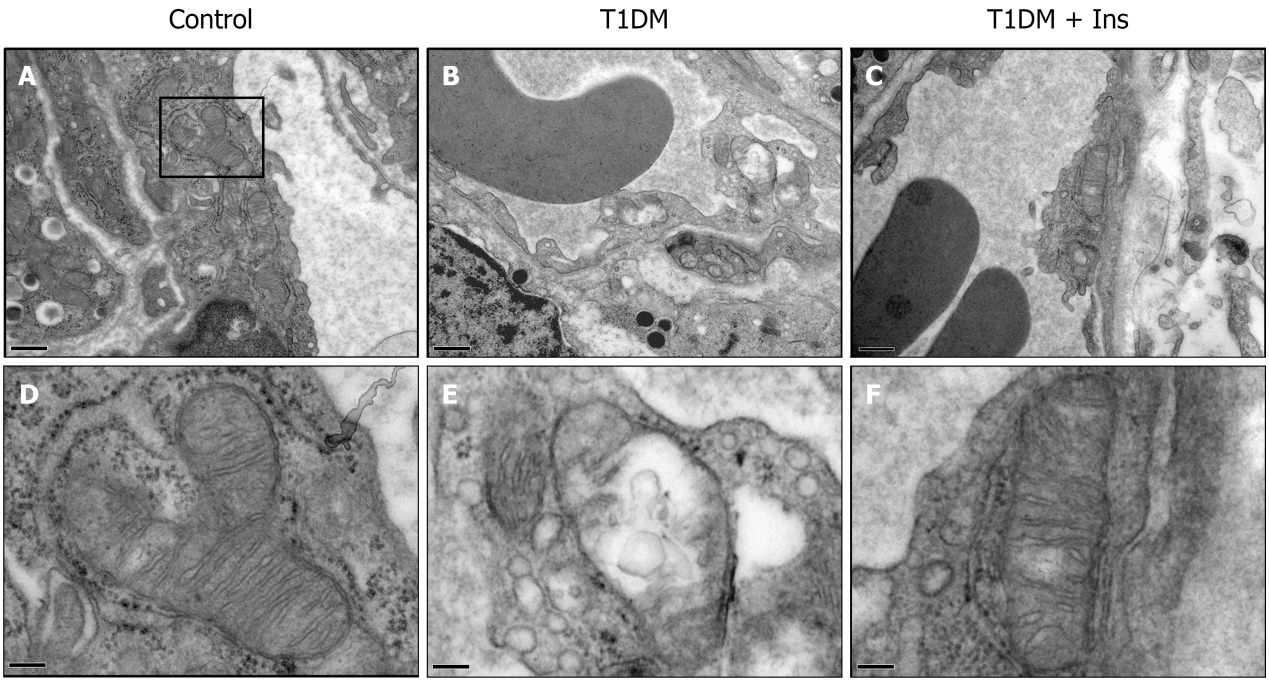
DOI: 10.4239/wjd.v13.i9.765

Figure 1 Integrated pancreatic microcirculatory oxygen profile. A-F: The pancreatic microcirculatory oxygen parameters of control, type 1 diabetes mellitus (T1DM) and insulin-treated mice were captured by probes of O2C and Microx TX3. Python and Apache ECharts were used to generate and visualize the three-dimensional (3-D) module of the integrated pancreatic microcirculatory oxygen profile; G-I: Comparisons of pancreatic microcirculatory oxygen profiles among groups. Partial pressure of oxygen, relative amount of hemoglobin, and hemoglobin oxygen saturation levels in control and T1DM mice with or without insulin administration are illustrated. $^{a}P < 0.05$, $^{b}P < 0.01$. Control, control mice; T1DM, STZ-induced T1DM mice without insulin administration; Insulin-treated, STZ-induced diabetic mice with 1.5 IU administration. T1DM: type 1 diabetes mellitus; Ins: Insulin; SO_2: Hemoglobin oxygen saturation; rHb: Relative amount of hemoglobin; PO_2: Partial pressure of oxygen; O2C: Oxygen to See.

indicate the basal glycolytic function when 10 mmol/L glucose was preadded into the medium before any pharmacological intervention. The ECAR-associated glycolytic capacity was subsequently reached after the injection of oligomycin. In this study, the values of both OCR and ECAR were normalized to 10^4 cells.

Statistical analysis

SPSS software 21.0 (IBM, Armonk, NY) was used to perform the statistical analyses. The data are shown as the mean ± standard error of the mean. The data sets were subjected to one-way ANOVA and post hoc multiple comparisons. A *P* value under 0.05 was considered to indicate statistical significance. In addition, the correlation between the microcirculatory oxygen profile and bioenergetics of IMECs was analyzed by Pearson's method.



DOI: 10.4239/wjd.v13.i9.765

Figure 2 Glucotoxicity induced ultrastructural damage to mitochondria in islet microvascular endothelial cells. The ultrastructure of pancreatic islet microvascular endothelial cells (IMECs) in the control (A), type 1 diabetes mellitus (T1DM) (B) and insulin-treated groups (C) was revealed by TEM (upper panels, scale bar = 0.5 μm). The ultrastructure of mitochondria in IMECs in the control (D), T1DM (E) and insulin-treated groups (F) is shown in the lower panels. Swollen mitochondria with cristae rupture or disappearance and a transparent matrix were found in T1DM mice. Restored mitochondria were observed after insulin administration (lower panels, scale bar = 2 μm).

RESULTS

Insulin ameliorates the decrease in the integrated microcirculatory oxygen profile

In this study, the efficiency of STZ to induce T1DM mice model was 100%. To analyze the integrated microcirculatory oxygen profile of islet microcirculation, the preprocessed raw data were imported into the common microcirculatory framework. The oscillation of the microcirculatory oxygen profile is shown in histograms of the 3-D module (Figure 1A-C), and the distribution pattern of the microcirculatory oxygen profile was indicated using a scatter plot (Figure 1D-F, Videos 1-6). Loss of microcirculatory oxygen was observed in T1DM mice, which exhibited a significant decrease in PO_2, rHb, and SO_2 compared with nondiabetic controls. Additionally, insulin administration ameliorated the STZ-induced decreases in these microcirculatory oxygen parameters (Figure 1G-I).

Insulin improves the mitochondrial ultrastructural abnormalities in IMECs in T1DM mice

Given that microvessels are responsible for distributing oxygen, we sought to determine whether the mitochondrial ultrastructure of IMECs changes in T1DM mice. TEM revealed the normal architecture of IMECs in the nondiabetic control group, with ovoid nuclei and well-arranged mitochondria in the cytoplasm. In contrast, mice with T1DM showed a narrowed or closed lumen with a contorted and thickened basement membrane, nuclear disaggregation, and mitochondrial swelling, suggesting an impaired ultrastructure of mitochondria in IMECs. The mitochondria in insulin-treated IMECs were restored, with a thin basement membrane, wide capillary lumen, and well-arranged parallel cristae (Figure 2). These data confirm the protective effect of insulin in the microcirculation of T1DM mice.

Effects of glucotoxicity and insulin administration on OCR and ECAR

The tight integration between endothelial metabolism and microcirculatory oxygen transport begs for integrative analysis that spans the cellular scale. We then performed real-time analysis of OCR and ECAR to determine energetic metabolic features in IMECs. The OCR of the IMECs was determined before and after receiving interventions of oligomycin, FCCP, and rotenone/antimycin A. A schematic of the real-time analysis of the IMEC OCR is depicted in Figure 3A. Our data revealed comparable mitochondrial maximal respiration in control, glucotoxicity-exposed, and insulin-treated IMECs, which was not induced after the injection of FCCP (Figure 3B).

Subsequently, to determine whether FCCP concentration is responsible for the evaluation of the MRC, the IMECs were incubated with different concentrations of FCCP (0.125, 0.25, 0.5, 1, and 2 μM) in the control and HG groups. Surprisingly, none of the OCR values exceeded the corresponding basal

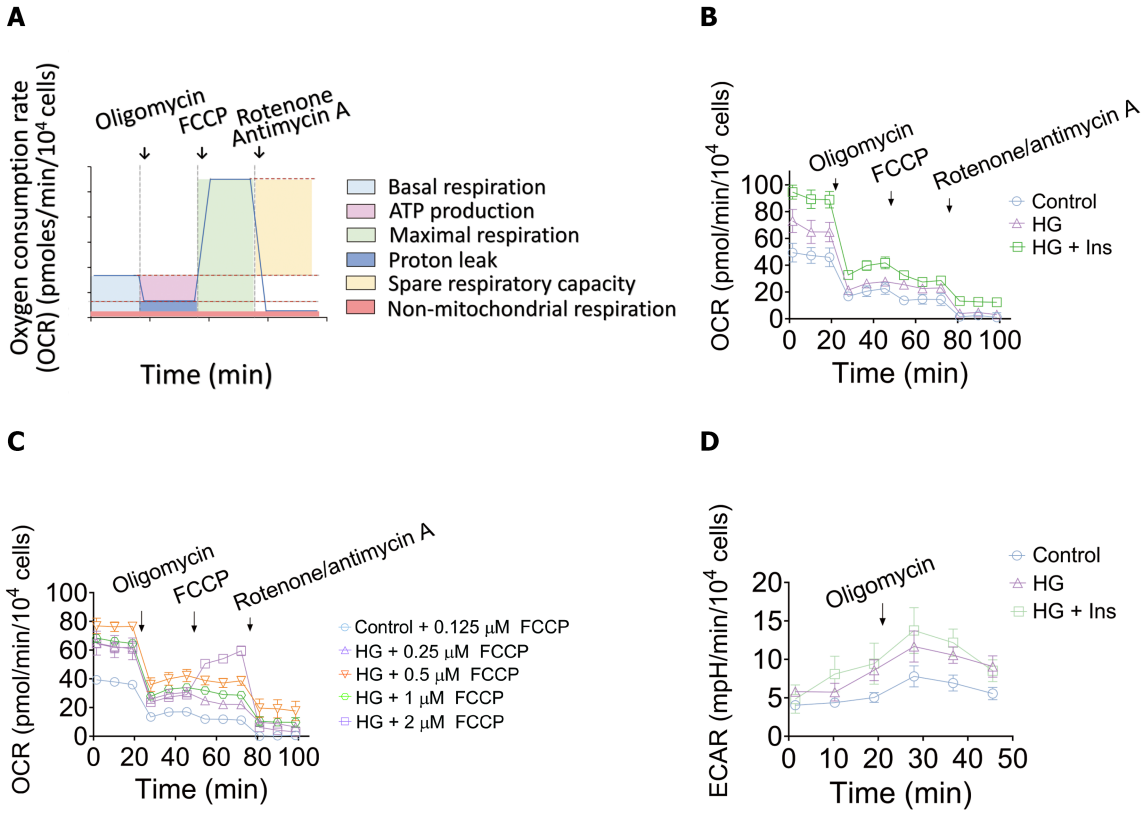


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Figure 3 Characterization of mitochondrial function in the control, glucotoxicity-exposed, and insulin-treated islet microvascular endothelial cell groups. A: Schematic representation of real-time mitochondrial respiration. The parameters of mitochondrial function were measured by kinetic oxygen consumption rate (OCR) analysis, starting from basal detection and after the injection of oligomycin (complex V inhibition), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, maximal respiration induction), and rotenone/antimycin A mixture (electron transport chain inhibition). Non-mitochondrial respiration was directly measured. Basal respiration, ATP production, maximal respiration, proton leak, and mitochondrial spare respiratory capacity were then calculated according to the OCR curve; B: Representative kinetic curve of mitochondrial OCR in control, glucotoxicity-exposed islet microvascular endothelial cells (IMECs) (HG), and insulin-treated IMECs (HG + Ins) after sequential injection of oligomycin, FCCP, and rotenone/antimycin A; C: Representative kinetic curve of mitochondrial OCR in control and glucotoxicity-exposed IMECs (HG) after sequential injection of gradient FCCP concentrations; D: Representative kinetic curve of extracellular acidification rate (ECAR) after injection of oligomycin. The peak values of ECAR reflect the glycolytic capacity. The data are presented as the mean ± SEM, $n = 4$ for each group. OCR: Oxygen consumption rate; FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; HG: High glucose; Ins: Insulin.

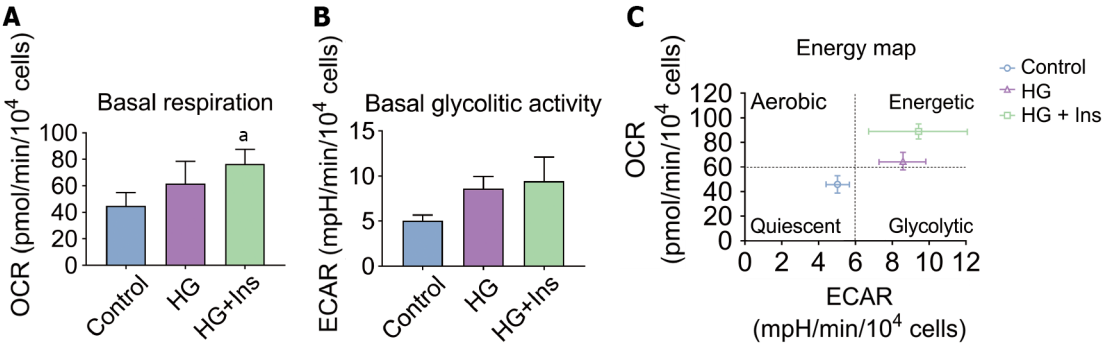
OCR after the FCCP injections, suggesting that the IMECs do not have SRC (Figure 3C). The ECAR values were simultaneously measured to reflect the glycolytic activity of IMECs. After 0.5 μM oligomycin injection, the glycolytic capacity was recorded as the peak value of ECAR (Figure 3D).

Insulin administration increases basal respiration and glycolytic capacity

Insulin-treated IMECs exhibited significantly increased basal respiration in comparison with glucotoxicity-exposed IMECs ($P < 0.05$, Figure 4A). However, there were no significant differences in the basal glycolytic activity among the groups (all $P > 0.05$, Figure 4B). Moreover, an energy map was plotted based on the basal respiration and glycolytic activity in the IMECs. IMECs in the control group were in the quiescent quadrant (lower left). Glucotoxicity-exposed IMECs shifted to the energetic quadrant (upper right), reflecting increased mitochondrial activity. Insulin-administered IMECs were located in the right upper energetic quadrant, revealing more energetic metabolism (Figure 4C), suggesting that insulin increased the glycolytic activity of glucotoxicity-exposed IMECs when needed.

Insulin administration activates oxidative metabolism and alleviates glucotoxicity-induced microcirculatory failure in IMECs

The basal respiration of mitochondria and non-mitochondrial respiration constitute oxidative metabolism in IMECs. Specific mitochondrial and non-mitochondrial functions were subsequently analyzed. ATP production, non-mitochondrial respiration, and oxidative metabolism were significantly increased in insulin-treated IMECs ($P < 0.05$, Figure 5A, D and E). However, proton leak (Figure 5B), coupling efficiency (Figure 5C), and endothelial glycolytic capacity (Figure 5F) were comparable among the groups.



DOI: 10.4239/wjd.v13.i9.765

Figure 4 Basal respiration and glycolytic activity in islet microvascular endothelial cells. The oxygen consumption rate and extracellular acidification rate were measured and compared among control, glucotoxicity-exposed islet microvascular endothelial cells (IMECs) (HG), and IMECs (HG + Ins). A: Basal respiration among groups; B: Basal glycolytic activity among groups; C: Glucotoxicity-exposed and insulin administered IMECs display distinct metabolic phenotypes. The data are presented as the mean ± SEM, $n = 4$ for each group. $^{a}P < 0.05$. OCR: Oxygen consumption rate; ECAR: Extracellular acidification rate; HG: High glucose; Ins: Insulin.

The correlation between the microcirculatory oxygen profile and bioenergetics of IMECs was then analyzed. Significant negative correlations between microcirculatory SO_2 and bioenergetic parameters (ATP production, basal respiration, oxidative metabolism, and non-mitochondrial respiration) were found by Pearson's correlation analysis (Figure 5G). These lines of evidence further confirmed that glucotoxicity in IMECs was related to pancreatic islet microcirculatory failure that could be reversed by insulin administration.

DISCUSSION

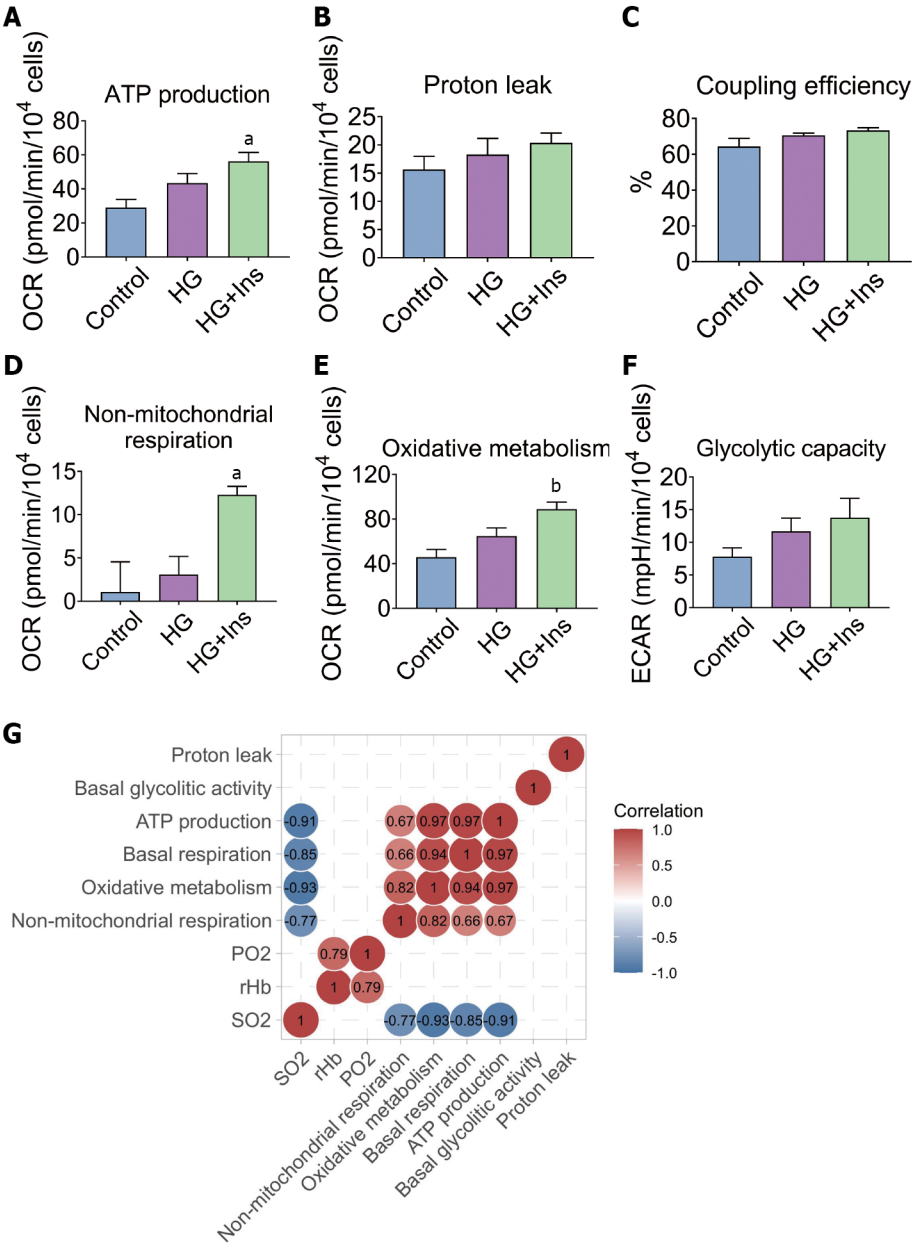
The influence of microcirculatory disturbance on the development of diabetes mellitus has been highlighted over decades[11]. However, the current data associated with pancreatic microcirculatory oxygen profiles are deficient. Here, we used a computer algorithm-based common microcirculatory framework to reveal integrated pancreatic microcirculatory oxygen profiles among groups. The existence of microcirculatory hypoxia in T1DM was noted.

Considering islet β cells, rather than IMECs, are specific target of STZ. Therefore, STZ-involved T1DM animal models are useful in elucidating the mechanisms of diabetic microvascular endothelial pathogenesis. IMECs are the key determinants in pancreatic islet microcirculation homeostasis. Blood perfusion and oxygen transport requires the coordinated communication of mitochondria with metabolic demands, which is influenced by a variety of factors (including hypoxia)[12]. Coinciding with the impairment in the microcirculatory oxygen profile, pathological alterations in mitochondrial ultrastructure and other subcellular structures have been observed in IMECs of T1DM mice. Earlier studies have reported that defects in mitochondrial function correlate with mal-matching adenosine triphosphate generation[13,14], which interferes with the bioenergetics of pancreatic islet microcirculation. Treatment with insulin during glucotoxicity exposure resulted in restoration of the ultrastructure of IMECs. Thus, our data suggest that insulin can improve the functional status of pancreatic islet microcirculation.

Metabolic capacity is important for energy regulation and the maintenance of cell survival[15]. In parallel with damage to the ultrastructure of IMECs, biogenetic mechanisms act during glucotoxicity exposure to compensate for the decreased blood perfusion and oxygen distribution. IMECs supplied with insulin increase their basal respiration and ATP production and switch to energetic adaptation. Mitochondria are important organelles for ATP production[16]. Dysfunction of mitochondria is one of the key determinants in the pathogenesis of diabetes[17].

Unexpectedly, our results indicated that maximal respiration of the mitochondria was not induced after injection of FCCP. Multiple factors are associated with FCCP-induced maximal respiration of mitochondria[18]. Therefore, to exclude the effect of FCCP concentration, we subsequently tested five FCCP concentrations, but none caused the basal OCR value to be exceeded, suggesting that the IMECs do not have SRC. In addition to the organ- and tissue-specific nature of microvascular endothelial cells [19], one of the possible explanations is that IMECs generate more than 85% of their ATP through glycolysis[20], which does not require an excessive number of mitochondria to obtain energy.

Furthermore, the increased OCR was associated with non-mitochondrial respiration, suggesting the existence of extensive ROS signaling caused by increased enzymatic activity of nitric oxide synthases, NADPH oxidase, and other oxygenases[21,22]. Although the glycolytic metabolism of endothelial cells is a protective strategy against oxidative stress[14], insulin can increase ROS production *via* activation of



DOI: 10.4239/wjd.v13.i9.765 **Copyright** ©The Author(s) 2022.

Figure 5 Metabolic characteristics of the islet microvascular endothelial cells. A-E: Oxygen consumption rates associated with mitochondrial ATP production, proton leak, coupling efficiency, non-mitochondrial respiration and oxidative metabolism; F: Endothelial glycolytic capacity evaluated by extracellular acidification rate; The data are presented as the mean ± SEM, $n = 4$ for each group. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control; G: The correlation analysis among pancreatic microcirculatory oxygen profile and microvascular endothelial mitochondrial metabolism. The correlation coefficients (r) in the control, glucotoxicity-exposed, and insulin-treated groups are illustrated as matrix plots. The numbers in the figure represent the correlation coefficient (r) values. SO_2: oxygen saturation; rHb, relative amount of hemoglobin; PO_2: partial oxygen pressure. OCR: Oxygen consumption rate; HG: High glucose; Ins: Insulin.

non-mitochondrial respiration *in vitro*. The excessive ROS levels and increased oxidative stress may lead to mitochondrial dysfunction[23] and endothelial dysfunction[24]. In this energy-demanding process, quiescent endothelial cells divide and migrate to form new vessels[25], and excessive ROS synthesis inhibits angiogenesis by inducing excessive ROS synthesis[26].

Similar to basal respiration, the basal glycolytic activity increases when insulin is present, although no significant difference was noted. An *in vitro* study indicated that insulin, in the context of high glucose, significantly activates oxidative metabolism other than glycolysis in IMECs, although endothelial cells are considered "glycolysis addicted"[27]. The OCR measurements for oxidative metabolism can be divided into three components, including OCR associated with ATP production, proton leak, and non-mitochondrial respiration; the first two indicators together constitute the basal respiration of the mitochondria. Increased ATP production-associated OCR was found in IMECs after insulin treatment, suggesting that mitochondrial energy metabolism participates in the regulatory effects of insulin on microvascular endothelial mitochondrial injury.

The unique role of mitochondria in endothelial cells implies that a cell-regulatory function other than their energy-providing function is dominant[28]. Our previous study indicated that the microvascular blood perfusion of pancreatic islets was significantly decreased in T1DM mice but was partially restored after the administration of insulin[4]. Negative correlations were observed between the microcirculatory oxygen profile and metabolic indexes in the control group. In addition, a relatively low level of mitochondrial metabolism was detected in glycolysis-addicted IMECs, suggesting that glucotoxicity broke the negative correlation due to decreases in microcirculatory perfusion and the oxygen profile.

The current study is the first report on the relationship between pancreatic microcirculatory oxygen profile and microvascular endothelial mitochondrial metabolism. However, there are still several limitations. First, the sample size of mice in each group was limited. Although pancreatic microcirculatory oxygen profile was measured at three random sites of the pancreas in each mouse, large sample size is preferred to ensure the data are representative. Second, in an interdependent functional relationship with β cells, IMECs are involved not only in the delivery of oxygen, but affect adult β cell function and promote β cell proliferation *via* vasoactive substances. However, the phenotypic and functional crosstalk between IMECs and islet β cells are not involved in our study.

CONCLUSION

In conclusion, glucotoxicity deteriorates the integrated pancreatic microcirculatory oxygen profile and bioenergetics, but this deterioration can be reversed by insulin administration.

ARTICLE HIGHLIGHTS

Research background

The pancreatic islet microcirculation adapts its metabolism to cope with limited oxygen availability and nutrient delivery. In diabetes, the balance between oxygen delivery and consumption is impaired. Insulin has been proven to exert complex actions promoting the maintenance of homeostasis of the pancreas under glucotoxicity.

Research motivation

We tried to provide new insight into the relationship between pancreatic microcirculatory oxygen profile and microvascular endothelial mitochondrial metabolism.

Research objectives

To test the hypothesis that insulin administration can improve the integrated pancreatic microcirculatory oxygen profile and bioenergetics.

Research methods

A three-dimensional framework was generated to visualize the pancreatic microcirculatory oxygen profile. The microcirculatory partial oxygen pressure (PO_2), relative hemoglobin (rHb) and hemoglobin oxygen saturation (SO_2) were evaluated in nondiabetic, type 1 diabetes mellitus (T1DM), and insulin-treated mice. An Extracellular Flux Analyzer was used to detect the real-time changes in bioenergetics by measuring the oxygen consumption rate and extracellular acidification rate in islet microvascular endothelial cells (IMECs).

Research results

Insulin administration ameliorated the glucotoxicity-induced decreases in microcirculatory oxygen parameters (PO_2, rHb, and SO_2) and improved the mitochondrial ultrastructural abnormalities in IMECs. Insulin-treated IMECs exhibited significantly greater basal respiration than glucotoxicity-exposed IMECs. An energy map revealed increased energetic metabolism in insulin-treated IMECs, with significantly increased ATP production, non-mitochondrial respiration, and oxidative metabolism. Significant negative correlations were revealed between microcirculatory SO_2 and bioenergetic parameters.

Research conclusions

Glucotoxicity deteriorates the integrated pancreatic microcirculatory oxygen profile and bioenergetics, but this deterioration can be reversed by insulin administration.

Research perspectives

Our understanding of the physiology and pathology of the pancreas islet microvascular endothelial cell in T1DM has been continually enhanced with the advancement of microcirculatory technology in

parallel with rapidly developing bioenergetics that allows us to increase resolution and precision in our investigations.

ACKNOWLEDGEMENTS

We thank Xi-Nan Duan for discussions about the computational algorithms.

FOOTNOTES

Author contributions: Liu MM designed the experiments; Li BW, Li Y, Zhang X, Fu SJ, Wang B, Zhang XY, Liu XT, Wang Q and Li AL performed the experiments; Li BW, Li Y and Liu MM analyzed the data; Li BW and Liu MM wrote the manuscript; Liu MM made critical revisions to the article for important intellectual content; All authors discussed the results and approved the final version of the manuscript.

Supported by the Beijing Municipal Natural Science Foundation, No. 7212068; the National Natural Science Foundation of China, No. 81900747.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at the Institute of Microcirculation, Chinese Academy of Medical Sciences & Peking Union Medical College.

Institutional animal care and use committee statement: All animal experiments conformed to the internationally accepted principles for the care and use of laboratory animals, and approved by the Institutional Animal Care and Use Committee at the Institute of Microcirculation, Chinese Academy of Medical Sciences & Peking Union Medical College.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 776-785

DOI: 10.4239/wjd.v13.i9.776

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Retrospective Study

Relationship between age of pregnant women with gestational diabetes mellitus and mode of delivery and neonatal Apgar score

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Elshazly AHM; Karalliedde J, United Kingdom; Ortega AL, Spain

Received: May 26, 2022
Peer-review started: May 26, 2022
First decision: June 20, 2022
Revised: July 1, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) refers to abnormal glucose tolerance during pregnancy, and it is often accompanied by obvious changes in glucose and lipid metabolism, and associated with adverse pregnancy outcomes. The incidence of fetal distress, polyhydramnios, puerperal infection, premature delivery, and macrosomia in pregnant women with GDM are higher than in those without GDM.

AIM

To analyze the relationship between age of pregnant women with GDM and mode of delivery and neonatal Apgar score.

METHODS

A total of 583 pregnant women with GDM who delivered in the Department of Obstetrics at our hospital between March 2019 and March 2022 were selected. Among them, 377 aged < 35 years were selected as the right age group and 206 aged > 35 years were selected as the older group. The clinical data of the two groups were collected, and the relationship between age of the pregnant women with GDM and mode of delivery, maternal and neonatal outcomes, and neonatal Apgar score were compared. In the older group, 159 women were classed as the adverse outcome group and 47 as the good outcome group according to whether they had adverse maternal and infant outcomes. The related factors of adverse maternal and infant outcomes were analyzed through logistic regression.

RESULTS

The number of women with assisted pregnancy, ≤ 37 wk gestation, ≥ 2 pregnancies, one or more deliveries, and no pre-pregnancy blood glucose screening in the older group were all higher than those in the right age group ($P < 0.05$). The natural delivery rate in the right age group was 40.85%, which was higher than 22.33% in the older group ($P < 0.05$). The cesarean section rate in the older group was 77.67%, which was higher than 59.15% in the right age group ($P < 0.05$). The older group had a higher incidence of polyhydramnios and postpartum hemorrhage, and lower incidence of fetal distress than the right age group had ($P < 0.05$). There was no significant difference in neonatal weight between the two groups ($P > 0.05$). The right age group had higher Apgar scores at 1 and 5 min than the older group had ($P < 0.05$). Significant differences existed between the poor and good outcome groups in age, education level, pregnancy mode, ≤ 37 wk gestation, number of pregnancies, and premature rupture of membranes ($P < 0.05$). Logistic regression showed that age, education level and premature rupture of membranes were all risk factors affecting the adverse outcomes of mothers and infants ($P < 0.05$).

CONCLUSION

Delivery mode and Apgar score of pregnant women with GDM are related to age. Older age increases the adverse outcome of mothers and infants.

Key Words: Gestational diabetes mellitus; Age; Mode of delivery; Neonatal Apgar score

Core Tip: This study analyzed the relationship between the age of pregnant women with gestational diabetes mellitus (GDM) and mode of delivery and neonatal Apgar score. Pregnant women with GDM were divided into right age and older groups. Compared with the older group, the natural delivery rate in the right age group was higher, but the cesarean section rate was lower. Moreover, age, education level and premature rupture of membranes were associated with the adverse outcomes of mothers and infants. Age was related to the delivery mode and Apgar score of pregnant women with GDM.

Citation: Gao L, Chen CR, Wang F, Ji Q, Chen KN, Yang Y, Liu HW. Relationship between age of pregnant women with gestational diabetes mellitus and mode of delivery and neonatal Apgar score. *World J Diabetes* 2022; 13(9): 776-785

URL: https://www.wjgnet.com/1948-9358/full/v13/i9/776.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i9.776

INTRODUCTION

Gestational diabetes mellitus (GDM) is one of the specific diseases of pregnant women. It refers to abnormal glucose tolerance in different degrees during pregnancy, often accompanied by obvious changes in glucose and lipid metabolism, and is closely related to adverse pregnancy outcomes. The incidence rate of GDM is increasing annually in China. GDM results in a high-risk pregnancy, which can induce complications, such as abortion, premature delivery, amniotic fluid and infection[1,2]. The incidence of fetal distress, polyhydramnios, puerperal infection, premature delivery, and macrosomia in pregnant women with GDM are higher than in those without GDM[3,4]. Pregnancy at the right age is the key to reduce the risk of adverse outcomes. With the implementation of China's three-child policy, the number of pregnant women with advanced maternal age is gradually increasing, and the incidence of pregnancy complications is significantly higher than that in right-age pregnant women. In recent years, with the rapid development of medical technology, significant progress has been made in the treatment of birth defects and premature infants. However, there are no effective measures to avoid the adverse outcomes of older-age pregnancies, especially those with GDM. Previous studies have shown that body mass index (BMI) may have an impact on maternal and neonatal outcomes in pregnant women with GDM[5,6]. However, pregnancy is still a risk factor for adverse maternal and neonatal outcomes and is affected by many factors. The clinical data of 583 pregnant women with GDM who delivered in the Department of Obstetrics at our hospital between March 2019 and March 2022 were retrospectively analyzed. The delivery mode and neonatal Apgar score of pregnant women with GDM at different ages were compared, to improve the pregnancy outcome of the older pregnant women, and provide a reference for ensuring the effect of eugenics and prenatal care.

MATERIALS AND METHODS

General data

The clinical data of 583 pregnant women with GDM who delivered in the Department of Obstetrics at our hospital between March 2019 and March 2022 were retrospectively analyzed. This study was approved by the Ethics Committee of our hospital. Inclusion criteria were: (1) All women conformed to the clinical diagnostic criteria for GDM[7]; (2) regular pregnancy examination; (3) successful delivery; (4) no hereditary diseases of coagulation system; (5) complete clinical medical records; and (6) pregnant women and family members gave informed consent to participate in this study.

Exclusion criteria were: (1) Women were diagnosed with diabetes mellitus or impaired glucose regulation before pregnancy; (2) women with other pregnancy-related diseases; (3) heart, liver or kidney dysfunction; (4) hematological diseases; (5) other diseases that may affect blood glucose; and (6) mental illness or retardation. Among the selected pregnant woman, 377 aged < 35 years were selected as the right age group and 206 aged > 35 years were selected as the older group. The data flow chart is shown in Figure 1.

Clinical data selection

The clinical data of the two groups were collected, including: age; pregnancy mode; educational level; BMI; fasting blood glucose; gestational weeks of delivery; number of pregnancies; number of deliveries; pre-pregnancy blood glucose screening; maternal and infant outcomes (preterm birth, polyhydramnios, oligohydramnios, fetal distress, macrosomia, umbilical cord around the neck, neonatal death events, neonatal hospitalization, neonatal aspiration pneumonia, neonatal hypoglycemia, neonatal jaundice, and postpartum hemorrhage); and Apgar scores at 1 and 5 min after birth. The clinical data of pregnant women in the two groups were retrospectively analyzed. In the older group, 159 women were classed as the adverse outcome group and 47 as the good outcome group according to whether they had adverse maternal and infant outcomes.

Diagnostic criteria

GDM was diagnosed by the International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria. The IADPSG recommends testing to be routinely carried out between 24 and 28 wk of gestation or at the first prenatal visit in high-risk women. Based on the results of a 75-g, 2-h oral glucose tolerance test, a woman was diagnosed with GDM when one or more of her plasma glucose concentrations were equivalent to or exceeded the following levels: fasting, 92 mg/dL; 1 h, 180 mg/dL; or 2 h, 153 mg/dL.

Statistical analysis

The data in this study were analyzed using SPSS 21.0. The measurement data were expressed as mean ± SD, and were compared using the independent sample *t* test between two groups. The enumeration data were expressed as *n* (%), and were compared using the χ^2 test between two groups. Factors related to adverse maternal and infant outcomes in older pregnant women were analyzed using logistic regression analysis. $P < 0.05$ was regarded as statistically significant.

RESULTS

Comparison of general data of pregnant women with GDM in two groups

The number of women with assisted pregnancy, ≤ 37 wk gestation, ≥ 2 pregnancies, one or more deliveries, and no pre-pregnancy blood glucose screening in the older group were all higher than those in the right age group ($P < 0.05$) (Table 1).

Comparison of delivery modes between the two groups

The right age pregnant women had a higher natural delivery rate of 40.85% compared with 22.5% in the older group ($P < 0.05$). The older group had a higher cesarean section rate of 77.67% compared with 59.15% in the right age group ($P < 0.05$) (Table 2).

Comparison of adverse outcomes between two groups

The older group had a higher incidence of polyhydramnios and postpartum hemorrhage, and lower incidence of fetal distress than the right age group had ($P < 0.05$) (Table 3).

Comparison of Apgar score between two groups

No significant difference existed in neonatal weight between the two groups ($P > 0.05$). The right age group had higher Apgar scores at 1 and 5 min after birth than the older group had ($P < 0.05$) (Table 4).

Table 1 Comparison of general data of pregnant women with gestational diabetes mellitus in two groups

General data		Right age group (n = 377)	Older group (n = 206)	t/χ^2	P
Pregnancy mode	Natural pregnancy	343 (90.98)	170 (82.52)	9.018	0.003
	Assisted pregnancy	34 (9.02)	36 (17.48)		
Education level	Primary school and below	4 (1.06)	5 (2.43)	5.054	0.080
	Junior high school	80 (21.22)	57 (27.67)		
	College degree or above	293 (77.72)	144 (69.90)		
BMI before pregnancy (kg/m^2)		22.30 ± 3.77	22.80 ± 3.75	1.534	0.126
Gestational weight gain (kg)		10.83 ± 15.21	9.20 ± 16.15	1.210	0.227
FBG (mmol/L)		4.93 ± 1.14	4.89 ± 0.69	0.460	0.646
≤ 37 wk gestation		110 (29.18)	84 (40.78)	8.072	0.004
No. of Pregnancies	1	134 (35.54)	24 (11.65)	38.493	< 0.001
	≥ 2	243 (64.46)	182 (88.35)		
Delivery times	0	226 (59.95)	59 (28.64)	52.441	< 0.001
	1	129 (34.22)	123 (59.71)		
	≥ 2	22 (5.84)	24 (11.65)		
Pre-pregnancy blood glucose screening	Yes	159 (42.18)	67 (32.52)	5.227	0.022
	No	218 (57.82)	139 (67.48)		

BMI: Body mass index; FBG: Fasting blood glucose.

Table 2 Comparison of delivery modes between the two groups

Groups	Cases	Natural delivery	Cesarean section rate
Right age group	377	154 (40.85)	223 (59.15)
Older group	206	46 (22.33)	160 (77.67)
χ^2		20.271	
P		< 0.001	

Analysis of related factors of adverse maternal and infant outcomes in older pregnant women

Significant differences existed between the poor (n = 159) and good (n = 47) outcome groups for age, education level, pregnancy mode, ≤ 37 wk gestation weeks, number of pregnancies, and premature rupture of membranes (P < 0.05) (Table 5).

Logistic regression analysis of risk factors for maternal and infant adverse outcomes in older pregnant women with GDM

The interference between the various indicators was controlled and the correlation between these indicators and maternal and infant adverse outcomes in older women with GDM was analyzed by logistic regression analysis. The analysis was conducted using significant factors in Table 5 as independent variables and adverse maternal and infant outcomes as the dependent variable. The regression model was established by selecting the indexes such as age, education level, pregnancy mode, ≤ 37 wk gestation, number of pregnancies, and premature rupture of membranes. Logistic regression showed that age, education level and premature rupture of membranes were risk factors for maternal and infant adverse outcomes in older women with GDM (P < 0.05) (Table 6 and nomogram analysis in Figure 2).

DISCUSSION

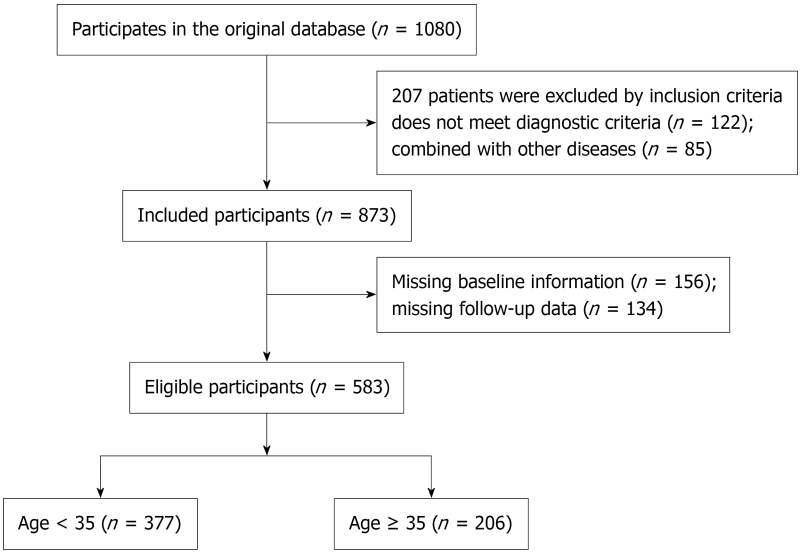
GDM is a special type of diabetes with a morbidity of 17.5%-18.9%, and the morbidity increases with

Table 3 Comparison of adverse outcomes between two groups

Adverse outcomes	Right age group (*n* = 377)	Older group (*n* = 206)	t/χ^2	*P*
Preterm birth	40 (10.61)	26 (12.62)	0.37	0.464
Polyhydramnios	5 (1.32)	8 (3.88)	3.996	0.046
Oligohydramnios	32 (8.49)	10 (4.85)	2.631	0.105
Fetal distress	40 (10.61)	10 (4.85)	5.628	0.018
Macrosomia	8 (2.12)	2 (0.97)	1.047	0.306
Umbilical cord around the neck	125 (33.16)	59 (28.64)	1.258	0.262
Neonatal death events	3 (0.80)	1 (0.49)	0.188	0.664
Neonatal hospitalization	4 (0.27)	1 (0.49)	0.519	0.471
Neonatal aspiration pneumonia	3 (0.80)	2 (0.97)	0.048	0.827
Neonatal Hypoglycemia	4 (0.27)	2 (0.97)	0.011	0.918
neonatal jaundice	5 (1.32)	2 (0.97)	0.142	0.706
Postpartum hemorrhage (mL)	318.62 ± 97.02	362.20 ± 175.92	3.861	0.001

Table 4 Comparison of Apgar score between the two groups

Groups	Cases	Neonatal weight (g)	1 min Apgar score	5 min Apgar score
Right age group	377	3107.66 ± 467.26	9.69 ± 0.06	9.93 ± 0.05
Older group	206	3102.07 ± 508.40	9.67 ± 0.08	9.89 ± 0.04
χ^2		0.134	3.408	9.882
P		0.894	0.001	< 0.001



DOI: 10.4239/wjd.v13.i9.776

Figure 1 Flow chart of general data selection.

age[8]. GDM increases the morbidity of pregnancy-related complications, which could lead to adverse pregnancy outcomes such as premature delivery, cesarean section, macrosomia and premature rupture of membranes, thus attracting the attention of the majority of medical staff and pregnant women[9,10]. Some scholars have found that gestational age > 35 years, pre-pregnancy BMI, and family history of diabetes are all risk factors for GDM, which increasing the incidence of adverse pregnancy outcomes such as premature delivery, macrosomia and fetal distress[11,12]. Previous studies have mainly focused on the high-risk factors of GDM[13-15], and have confirmed that advanced age is a risk factor for GDM.

Table 5 Analysis of related factors of adverse maternal and infant outcomes in older pregnant women

Factors		Poor outcome group (*n* = 112)	Good outcome group (*n* = 94)	t/χ^2	*P*
Age		38.75 ± 1.26	37.26 ± 1.78	7.011	< 0.001
Education level	Primary school and below	0 (0.00)	5 (5.32)	6.257	0.044
	Junior high school	33 (29.46)	24 (25.53)		
	College degree or above	79 (70.54)	65 (69.15)		
Pregnancy mode	Natural pregnancy	87 (77.68)	83 (88.30)	3.996	0.046
	Assisted reproduction	25 (22.32)	11 (11.70)		
≤ 37 wk gestation		53 (47.32)	31 (32.98)	4.354	0.037
No. of pregnancies	1	18 (16.07)	6 (6.38)	4.661	0.031
	≥ 2	94 (83.93)	88 (93.62)		
No. of deliveries	0	36 (32.14)	23 (24.47)	1.543	0.462
	1	63 (56.25)	60 (63.83)		
	≥ 2	13 (11.61)	11 (11.70)		
Mode of delivery	Natural labor	25 (22.32)	21 (22.34)	0.000	0.997
	Cesarean section	87 (77.68)	73 (77.66)		
Premature rupture of membranes		36 (32.14)	0 (0.00)	36.613	< 0.001

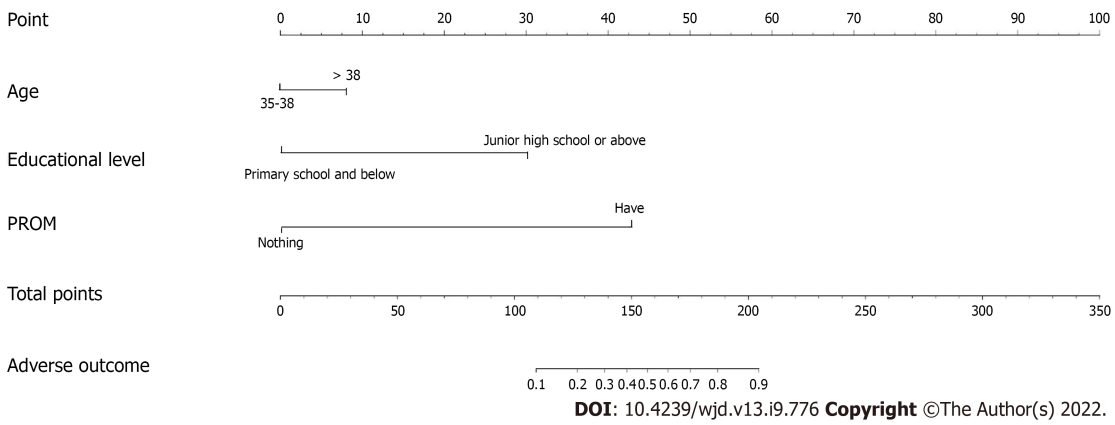
Table 6 Logistic regression analysis of risk factors for maternal and infant adverse outcomes in elderly pregnant women with gestational diabetes mellitus

Risk factors	B	SE	Wald χ^2	*P*	OR	95%CI
Age	0.485	0.074	15.090	0.005	1.254	1.002-4.056
Education level	0.650	0.112	20.482	0.019	1.234	1.051-5.573
Pregnancy mode	0.253	0.145	2. 774	0.045	1.254	0.976-1.780
≤ 37 wk gestation	0.504	0.256	3.157	0.086	1.643	0.949-2.954
No. of pregnancies	0.784	0.165	5.48	0.097	1.262	0.758-1.985
Premature rupture of membranes	0.864	0.142	16.751	0.011	1.318	1.185-9.254

SEM: Standard error of mean; OR: Odd ratio; CI: Confidence interval.

However, there has been less research on older women with GDM. In this study, we retrospectively analyzed the clinical data of older and right-age pregnant women with GDM. The general situation, delivery mode, and maternal and neonatal outcomes were compared, and the delivery characteristics of women with GDM at different ages were discussed, aiming to improve pregnancy outcome in the older age group and providing a reference for ensuring the effect of eugenics in China.

The number of women with assisted pregnancy, ≤ 37 wk gestation, ≥ 2 pregnancies, one or more deliveries, and no pre-pregnancy blood glucose screening in the older group were all higher than those in the right-age group. The ovarian function of older women decreased significantly, and the ovarian reserve and egg quality decreased gradually, resulting in a decline in successful pregnancy rate. Assisted reproductive technology helps a large number of infertile older women conceive successfully, but the complications during pregnancy are higher than those of pregnant women who conceive naturally. With the implementation of the three-child policy in China, the number of older pregnant women is increasing gradually. Older women have a higher incidence of pregnancy complications, such as GDM, and higher risk of maternal and infant deaths than pregnant women at the right age have. Therefore, we should attach importance to the healthcare of older women during pregnancy and in the perinatal period. The results of the present study showed that the older group had a higher cesarean section rate of 77.67%, compared with 59.15% in the right-age group. The right age group had higher Apgar scores at 1 and 5 min after birth compared with the older group. In recent years, the rate of cesarean section has increased due to incorrect fetal position or prevention of fetal distress. There are many pregnancy complications in older women, uterine contraction is weaker, and the total labor



DOI: 10.4239/wjd.v13.i9.776

Figure 2 Nomogram analysis of risk factors for adverse maternal and infant outcomes in older pregnant women. PROM: Premature rupture of membrane.

process is prolonged. Therefore, natural delivery can increase maternal and neonatal risks. This is one of the reasons why older women choose cesarean section, but this increases the risk of maternal and neonatal infection and thromboembolism[8,16-18]. With increasing age, pregnant women are prone to obesity, and various bodily functions gradually decrease. In older pregnant women with GDM, the incidence of fetal macrosomia is high. We showed that older women had a higher incidence of polyhydramnios and postpartum hemorrhage than the right-age women had, which confirms that GDM is closely related to maternal and infant adverse outcomes. According to previous studies, older age pregnancy has a higher risk of GDM[19-21]. Older age can also increase the probability of gestational hypertension, perinatal complications and other diseases. The present study found that the older group had a lower incidence of fetal distress than the right-age group had, which may be related to the high rate of cesarean section in the older group. The incidence of fetal distress was reduced due to the high proportion of women with ≤ 37 wk gestation in the older group.

To control the interference between the various indicators and analyze the correlation between related indicators and maternal and infant adverse outcomes in older women with GDM, logistic regression analysis was conducted. Logistic regression showed that age, education level and premature rupture of membranes were risk factors for maternal and infant adverse outcomes in older women with GDM. Pregnant women aged > 35 years old are more likely to have pregnancy complications during pregnancy or delivery. Laura estimated the risk of adverse outcome over a pregnancy cycle of 3-24 mo based on the maternal age at the initial birth (20-34 years and ≥ 35 years)[20]. The risk of maternal mortality or serious morbidity at 6 mo of pregnancy was increased compared to the 18-mo pregnancy cycle of women aged ≥ 35 years. Women aged 20-34 years had an increased risk of spontaneous preterm birth within 6 mo of pregnancy. In the present study, with the increase in academic qualifications, the proportion of maternal and infant adverse outcomes in older pregnant women with GDM increased, and there was a significant difference compared with the good outcome group. Education level is a risk factor for maternal and infant adverse outcomes in older women with GDM. The reason may be that the higher the educational background is, the later the childbearing age is, which affects fertility and reproductive quality. Premature rupture of membranes induces serious adverse effects in both the mother and fetus. In premature rupture of membranes, the reproductive tract loses its protective barrier, and the amniotic fluid gradually decreases, which affects the blood circulation of the placenta and increases proneness to adverse outcomes such as fetal distress[22,23]. Obstetric medical staff should focus on monitoring pregnant women with high-risk factors of premature rupture of membranes and implement timely intervention measures.

The limitation of this study was that the subjects were from a single institution, which limits the extrapolation of research results. More eligible samples will be included in future studies and more disease-related data will be analyzed, to enhance the reliability and validity of the results and provide a basis for treatment.

CONCLUSION

In summary, the delivery mode and neonatal Apgar score are related to the age of pregnant women with GDM and advanced age increases the adverse outcomes in mothers and infants. Therefore, to improve the pregnancy outcome and reduce the incidence of complications in pregnant women with GDM, it is suggested that pregnant women with a family planning plan should have pre-pregnancy eugenics health examination and pregnancy health care.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) is one of the serious pregnancy complications, which severely threatens the health of pregnant women and newborns. In recent years, with the increased childbearing age and proportion of overweight people, the incidence of GDM has an upward trend. The detrimental effect of GDM on the prognosis has been recognized, which can increase the incidence of dystocia, cesarean section and macrosomia, and 17%-63% of pregnant women and infants will develop type 2 diabetes in the long term.

Research motivation

We analyzed the risk factors affecting the adverse outcomes of mothers and infants, we also put forward targeted prevention and control measures to provide reference for formulating GDM early prevention and intervention policies.

Research objectives

To explore the relationship between the age of GDM pregnant women and the delivery mode and neonatal Apgar score, so as to provide a theoretical basis for reducing the incidence of adverse pregnancy outcomes.

Research methods

We used the latest diagnostic criteria of GDM to investigate pregnant women who met the inclusion criteria, and collected their general conditions before and during pregnancy and related clinical data. The women were divided into right age group and older group according to whether they were older than 35 years old. Logistic regression analysis was used to analyze the related risk factors affecting the delivery outcome of GDM pregnant women.

Research results

The older group had a higher cesarean section rate, higher incidence of polyhydramnios and postpartum hemorrhage, and lower incidence of fetal distress than the right age group. The right age group had higher Apgar scores at 1 and 5 min after birth than the older group. Moreover, age, education level and premature rupture of membranes were risk factors for adverse pregnant outcomes in older GDM women. Our results showed that gestational age greater than 35 years old will increase the incidence of gestational diabetes and adverse pregnancy outcomes, but it needs to be confirmed by large samples and studies involving patients in multiple research centers.

Research conclusions

The age of pregnant women with GDM affects the delivery mode and neonatal Apgar score, and advanced age increases the adverse pregnancy outcomes. Therefore, it is suggested that pregnant women should have pre-pregnancy eugenics health examination and pregnancy health care.

Research perspectives

In the future research, we want to explore the effective means of screening GDM in pregnancy physical examination, as well as the means of early intervention and treatment for pregnant women with high-risk factors of GDM.

FOOTNOTES

Author contributions: Gao L and Chen CR contributed equally to this work; Gao L and Chen CR drafted the manuscript; Yang Y provision patients; Gao L, Wang F, Ji Q collected the data; Chen CR and Liu HW analyzed and interpreted the data; Liu HW contributed to conception and design; Chen KN contributed to administrative support.

Supported by Hainan Province Clinical medical center and The High-level Talent Project of Hainan Provincial Natural Science Foundation, No.821RC685.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Hainan General Hospital.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All authors declare no conflict of interest.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Chang KL
L-Editor: A
P-Editor: Chang KL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 786-798

DOI: 10.4239/wjd.v13.i9.786

ISSN 1948-9358 (online)

SCIENTOMETRICS

Mapping the global research landscape on insulin resistance: Visualization and bibliometric analysis

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Balbaa ME, Egypt; Dabravolski SA, Belarus; LI L, China; Zeng Y, China

Received: March 3, 2022
Peer-review started: March 3, 2022
First decision: April 17, 2022
Revised: May 4, 2022
Accepted: August 5, 2022
Article in press: August 5, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Insulin resistance is a risk factor for metabolic syndromes and is associated with a wide variety of metabolic illnesses, including obesity, type 2 diabetes, and cardiovascular disease.

AIM

To investigate and map global insulin resistance studies.

METHODS

A bibliometric methodology was applied to the literature retrieved from the Scopus database and *Reference Citation Analysis* (https://www.referencecitationanalysis.com) by using a validated search strategy. The study period was limited from 2002 to 2021. Bibliometric indicators and mapping were presented.

RESULTS

A total of 26808 articles on the topic of insulin resistance were included in the Scopus database. The articles included research articles (n = 21918; 81.76%), review articles (n = 2641; 9.85%), and letters (n = 653; 2.44%). During the study period, 136 countries contributed to the research on insulin resistance. The highest number of articles was from the United States (n = 7360; 27.45%), followed by China (n = 3713; 13.85%), Japan (n = 1730, 6.45%), Italy (n = 1545; 5.54%), and the United Kingdom (n = 1484; 5.54%). The retrieved articles identified two main research themes: "inflammatory mechanisms in the regulation of insulin resistance" and "mechanisms linking obesity to insulin resistance".

CONCLUSION

Our data show that insulin resistance has steadily gained interest from researchers, as evidenced by the number of citations and yearly publications. Publications have grown significantly in the last decade, while low-income countries with greater burdens continue to produce fewer publications in this field. This approach might assist researchers in choosing new research areas and recognizing research hotspots and frontiers. In the future, perhaps high-quality clinical evidence will be acquired.

Key Words: Insulin resistance; Research hotspots; Scopus; VOSviewer; Bibliometric

Core Tip: Several bibliometric studies have been conducted in the field of diabetes research. However, no bibliometric study has been conducted on insulin resistance research. Therefore, the current study aims to investigate and map global research on insulin resistance. The retrieved articles identified two main research themes: "inflammatory mechanisms in the regulation of insulin resistance" and "mechanisms linking obesity to insulin resistance". This approach might assist researchers in choosing new research areas and recognizing research hotspots and frontiers. In the future, perhaps high-quality clinical evidence will be acquired.

Citation: Zyoud SH, Shakhshir M, Koni A, Abushanab AS, Shahwan M, Jairoun AA, Al Subu R, Abu Taha A, Al-Jabi SW. Mapping the global research landscape on insulin resistance: Visualization and bibliometric analysis. *World J Diabetes* 2022; 13(9): 786-798
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/786.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.786

INTRODUCTION

During the last two decades, the global prevalence of diabetes has increased dramatically. Diabetes is increasing worldwide, both in terms of prevalence and the number of affected[1]. For more than half a century, insulin resistance and type 2 diabetes have been associated. Insulin resistance is not only a powerful predictor of future type 2 diabetes development but is also a therapeutic target in the presence of hyperglycemia[2]. Insulin resistance is defined as a reduced physiological response to insulin stimulation of target tissues, especially adipose tissue, liver, and muscle. Insulin resistance limits glucose disposal, leading to a compensatory increase in beta cell insulin synthesis and hyperinsulinemia

[3]. More than 30 years ago, hyperinsulinemia and insulin resistance were hypothesized to be key contributors to hypertension, hyperglycemia, dyslipidemia, hyperuricemia, visceral adiposity, elevated inflammatory markers, prothrombic state, and endothelial dysfunction related to obesity and the metabolic syndrome[4].

Several bibliometric studies have been conducted in diabetes research[5-9] or in depression and insulin research[10]. However, no bibliometric study has been conducted on insulin resistance research. As a scientific evaluation approach, bibliometrics can assess the research impact of organizations and individuals[11]. Similarly, bibliometrics provide evidence to promote the formation of future research hotspots[12,13]. As a result, this research aims to examine the scientific development in insulin resistance thoroughly. Therefore, this bibliometric analysis was designed to examine the research trend related to insulin resistance and identify future research hotspots. Furthermore, the study offers some important information by providing references and ideas for future studies on insulin resistance pathophysiology and clinical applications.

MATERIALS AND METHODS

Data acquisition

The documents in the current study were obtained and downloaded from the Scopus database on January 29, 2022 to prevent bias caused by the database's daily updates. With more than 36000 titles from around 11678 publishers, of which 34346 were peer-reviewed journals, Scopus is one of the most extensive and authoritative databases for collecting academic information[14,15]. Unfortunately, only one database may be utilized in bibliometric analyses because data from many databases cannot be integrated and analyzed. On the other hand, systematic reviews use multiple databases to retrieve a large number of documents for further analysis[16]. Furthermore, only one database was chosen on the topic and objective coverage, and past research has shown that Web of Science and PubMed are included in the Scopus database. Based on previous studies and findings, it was recommended to use Scopus (Elsevier database) because it was the most comprehensive database on the subject, offering all the data needed for quantitative analysis[17,18].

Search strategy

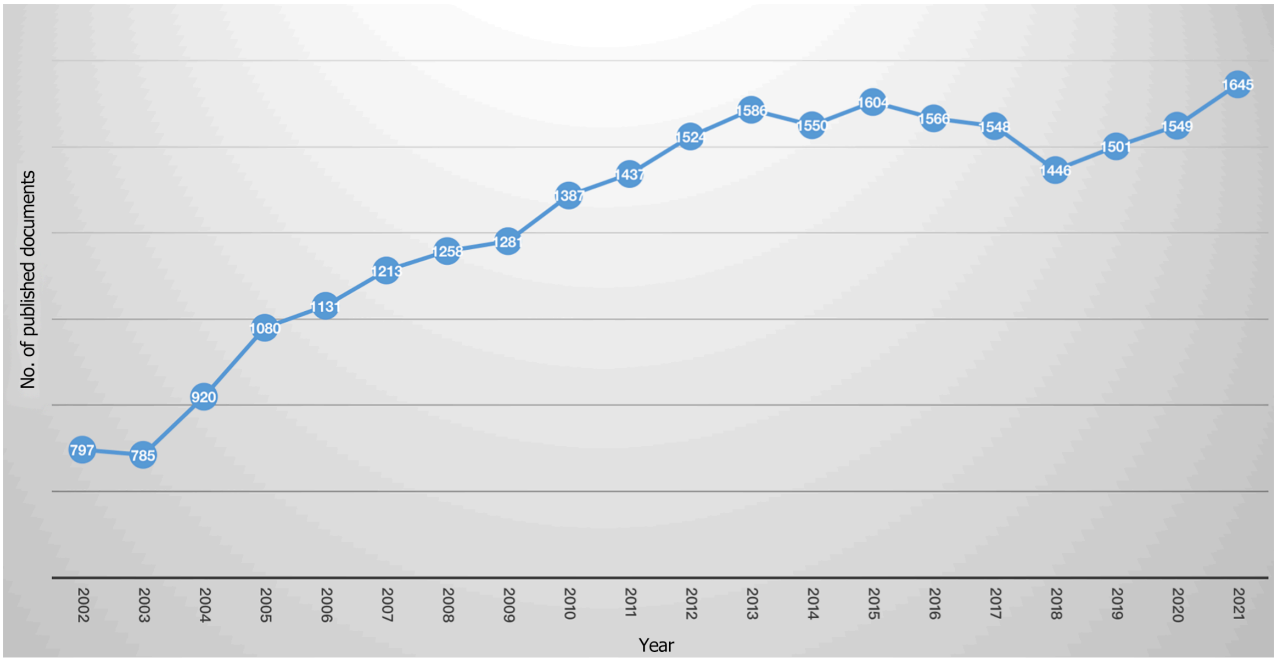
Keywords used in the Scopus engine to achieve the aim of this study were chosen from previous systematic reviews and meta-analyses on insulin resistance[19-21]. "Insulin resistance" or "insulin sensitivity" was used as a search expression in the title search in the Scopus database over the last two decades (January 2002 to December 2021). This study used the keywords "insulin resistance" or "insulin sensitivity" because we are more interested in these terms than related terminology. Therefore, keywords were used instead of a title/abstract search in the title search. Consequently, the search for the title will provide the fewest false positive documents, making it a trustworthy strategy[22-26]. A title/abstract search, on the other hand, will provide numerous false positives in which the main focus is not on insulin resistance per se.

Bibliometric analysis

As described in previous studies, the bibliometric technique was applied[27-30]. The following bibliometric indicators were generated when the refined findings were exported to Microsoft Excel: (1) Growth pattern; (2) Type of publications; (3) Core countries; (4) Core institutions; (5) Core funding agencies; (6) Prolific authors; (7) Core journals with their impact factors (IF); and (8) Top 10 cited articles. The Impact Index per article for the top 10 highly-cited papers collected from *Reference Citation Analysis*, https://www.referencecitationanalysis.com, was presented. *Reference Citation Analysis* is an open, multidisciplinary citation analysis database owned by Baishideng Publishing Group Inc. (Pleasanton, CA 94566, United States)[31].

Visualized analysis

VOSviewer 1.6.18 was used to perform a co-occurrence analysis and visualize the collaborative networks of the countries to determine a worldwide scientific cooperation network across countries/regions and keywords in the titles and/or abstracts to determine hotspots and research trends. VOSviewer maps have nodes or frames that are colored and scaled differently. The node or the frame size is proportional to the number of times it appears. The node's or the frame's color indicates its link to other nodes with similar colors[32].



DOI: 10.4239/wjd.v13.i9.786

Figure 1 Annual growth of publications on insulin resistance research the last two decades (2002-2021). Source: Own elaboration, based on Scopus; this figure created using EXCEL version 2013.

RESULTS

Current status and annual trend

A total of 26808 articles on insulin resistance were included in the Scopus database. The articles included research articles (n = 21918; 81.76%), review articles (n = 2641; 9.85%), and letters (n = 653; 2.44%). After 2003, as shown in Figure 1, the number of publications on insulin resistance studies increased rapidly. In 2021, 1645 papers were published, the highest amount in two decades.

Analysis of countries

During the study period, 136 countries contributed to research on insulin resistance. The highest number of articles was from the United States (n = 7360; 27.45%), followed by China (n = 3713; 13.85%), Japan (n = 1730, 6.45%), Italy (n = 1545; 5.54%), and the United Kingdom (n = 1484; 5.54%) (Table 1). The country network map included 42 frames (Figure 2). The top three countries in terms of centrality were the United States, China, and the United Kingdom. The centrality proved that they had close relationships and substantial intellectual effects on other countries.

Analysis of institutions

The top 10 active institutions are listed in Table 2. Harvard Medical School was first with 515 (1.92%) articles, followed by INSERM with 451 (1.68%) articles and the National Institutes of Health with 298 (1.11%). The top 10 active institutions were mainly based in the United States.

Analysis of funding agencies

Table 3 lists the top 10 funding agencies with the highest output. Seven funding agencies are from the United States, and one each is from Japan, China, and Canada. These countries contributed 10459 (39.01%) documents. The three most productive funding agencies were the National Institute of Diabetes and Digestive and Kidney Diseases (n = 2548; 9.50%), the National Institutes of Health (n = 2094, 7.81%), and National Heart, Lung, and Blood Institute (n = 1140, 4.25%).

Analysis of journals

Table 4 shows the top 10 most active journals. Diabetes Journal was first (n = 830; 3.10%), followed by Clinical Endocrinology and Metabolism (n = 692, 2.58%) and Diabetes Care (n = 623; 2.32%). Four of the journals on the active list were on the subject of diabetes. All the journals on the active list have a relatively high impact factor.

Analysis of citations

Table 5 lists the top 10 articles that were the most cited in research related to insulin resistance from 2002

Table 1 Top 10 most productive countries on insulin resistance research, ranked by the total number of publications in the last two decades (2002-2021)

Ranking	Country	Number of documents	%
1st	United States	7360	27.45
2nd	China	3713	13.85
3rd	Japan	1730	6.45
4th	Italy	1545	5.76
5th	United Kingdom	1484	5.54
6th	Canada	1186	4.42
7th	Germany	1070	3.99
8th	Spain	1061	3.96
9th	South Korea	1056	3.94
10th	France	858	3.20

Table 2 Top 10 most productive institutions in insulin resistance research, ranked by the total number of publications in the last two decades (2002-2021)

Ranking	Institute	Country	*n*	%
1st	Harvard Medical School	United States	515	1.92
2nd	INSERM	France	451	1.68
3rd	National Institutes of Health	United States	298	1.11
4th	University of Toronto	Canada	286	1.07
5th	Københavns Universitet	Denmark	280	1.04
6th	Karolinska Institutet	Sweden	268	1.00
7th	Consiglio Nazionale delle Ricerche	Italy	263	0.98
8th	VA Medical Center	United States	253	0.94
9th	Universidade de São Paulo	Brazil	247	0.92
10th	Yale School of Medicine	United States	234	0.87

to 2021. The 10 highest citations ranged from 4911 to 1827[33-42]. Furthermore, the 10 most cited articles have an impact index per article of 101.5 to 241.2 (Table 5).

Term co-occurrence cluster analysis of research hotspots

The term co-occurrence analysis provided a complete summary of hot topics discussed in insulin resistance research. VOSviewer detected 456 keywords that appeared a minimum of 300 times in the titles and abstracts of the included articles by analyzing the contents of the titles and abstracts. All terms were sorted into clusters on the VOSviewer keyword co-occurrence visualization map, and various clusters were colored differently (Figure 3). There are two clusters: (1) Cluster #1, shown by green dots, contained phrases typically found in publications relating to "inflammatory mechanisms in the regulation of insulin resistance"; and (2) Cluster #2, shown by red dots, contained phrases typically found in publications relating to "mechanisms linking obesity to insulin resistance". Hotspots in the field of insulin resistance were revealed *via* an overlay visualization map scaled by occurrence. The colored terms differ depending on when they appeared in the literature. The blue keywords were first shown, followed by the yellow keywords. After 2013, the most popular terms were related to inflammatory mechanisms in the regulation of insulin resistance (Figure 4).

Analysis of authorship

The total number of authors who participated in the publication of the retrieved documents was 80932, a mean of 3.1 authors per document. The list of the top 10 active authors in insulin resistance research, ranked by the total number of publications in the last two decades (2002-2021), is shown in Table 6. The top 10 list included four from the United States, three from Germany, two from Spain, and one from Italy.

Table 3 The top 10 funding agencies having the most publications on insulin resistance, ranked by the total number of publications in the last two decades (2002-2021)

Ranking	Institute	Country	*n*	%
1st	National Institute of Diabetes and Digestive and Kidney Diseases	United States	2548	9.50
2nd	National Institutes of Health	United States	2094	7.81
3rd	National Heart, Lung, and Blood Institute	United States	1140	4.25
4th	National Natural Science Foundation of China	China	1137	4.24
5th	National Center for Research Resources	United States	1051	3.92
6th	United States Department of Health and Human Services	United States	629	2.35
7th	National Institute on Aging	Canada	521	1.94
8th	Japan Society for the Promotion of Science	Japan	466	1.74
9th	National Center for Advancing Translational Sciences	United States	450	1.68
10th	Eunice Kennedy Shriver National Institute of Child Health and Human Development	United States	423	1.58

Table 4 Top 10 most productive journals on insulin resistance research, ranked by the total number of publications in the last two decades (2002-2021)

Ranking	Journal	*n*	%	IF[1]
1st	Diabetes	830	3.10	9.461
2nd	Journal of Clinical Endocrinology and Metabolism	692	2.58	5.958
3rd	Diabetes Care	623	2.32	19.112
4th	Plos One	517	1.93	3.2400
5th	Diabetologia	499	1.86	10.122
6th	Clinical and Experimental	425	1.59	8.694
7th	American Journal of Physiology Endocrinology and Metabolism	377	1.41	4.310
8th	Diabetes Research and Clinical Practice	227	0.85	5.602
9th	Obesity	219	0.82	5.002
10th	Scientific Reports	218	0.81	4.379

[1]2020 Journal Citation Reports® Science Edition (Clarivate Analytics, 2021). IF: Impact factor

DISCUSSION

Bibliometric analysis of insulin resistance publications in the last 20 years revealed that the number of articles published has gradually increased in recent years, indicating that more and more researchers are becoming involved in insulin resistance research. To our knowledge, this is the first bibliometric study that comprehensively examined worldwide trends in insulin resistance research over the last 20 years. The current study showed that research activity on insulin resistance was worldwide and involved countries in different world regions. The United States and China had a noticeable edge on this topic, probably due to a greater economy and investment in the scientific field. The research output from these countries may be related to a diverse spectrum of researchers interested in this topic and strong financial support for researchers.

Another important reason for the contribution of different world regions is the high level of international collaboration, as evident from the thick lines coming out from most countries in the visualization map. This collaboration was initiated because different regions of the research groups in different regions of the world were involved in different aspects of insulin resistance research or different complications of insulin resistance. Another area of relevance for the current study with regard to scientific publications on insulin resistance is the quality of research papers. It is worth noting that nine of the top 10 cited articles were published in journals with an IF larger than 10, implying that they have a large impact in medicine: Journal of Clinical Investigation, Cell Metabolism, and Nature. As shown, articles related to insulin resistance have been published both in endocrinology and non-endocrinology

Table 5 Top 10 most cited papers on research related to insulin resistance, ranked by the total number of citations in the last two decades (2002-2021)

Ranking	Ref.	Journal name	Cited by	IF[1]	Impact index per article[2]	Type of paper
1st	Xu *et al*[36], 2003	Journal of Clinical Investigation	4911	14.808	241.2	Original article
2nd	Cani *et al*[40], 2007	Diabetes	3645	9.461	222.2	Original article
3rd	Kahn *et al*[39], 2006	Nature	3109	49.962	185.2	Review articles
4th	Shoelson *et al*[37], 2006	Journal of Clinical Investigation	2822	14.808	156.4	Review articles
5th	Shi *et al*[42], 2006	Journal of Clinical Investigation	2521	14.808	149.0	Original article
6th	Hirosumi *et al*[35], 2002	Nature	2503	49.962	112.6	Letter to the editor
7th	Kadowaki *et al*[33], 2006	Journal of Clinical Investigation	2140	14.808	112.9	Review articles
8th	Newgard *et al*[34], 2009	Cell Metabolism	1852	27.787	139.7	Original article
9th	Houstis *et al*[41], 2006	Nature	1838	49.962	101.5	Letter to the editor
10th	Kanda *et al*[38], 2006	Journal of Clinical Investigation	1827	14.808	105.4	Original article

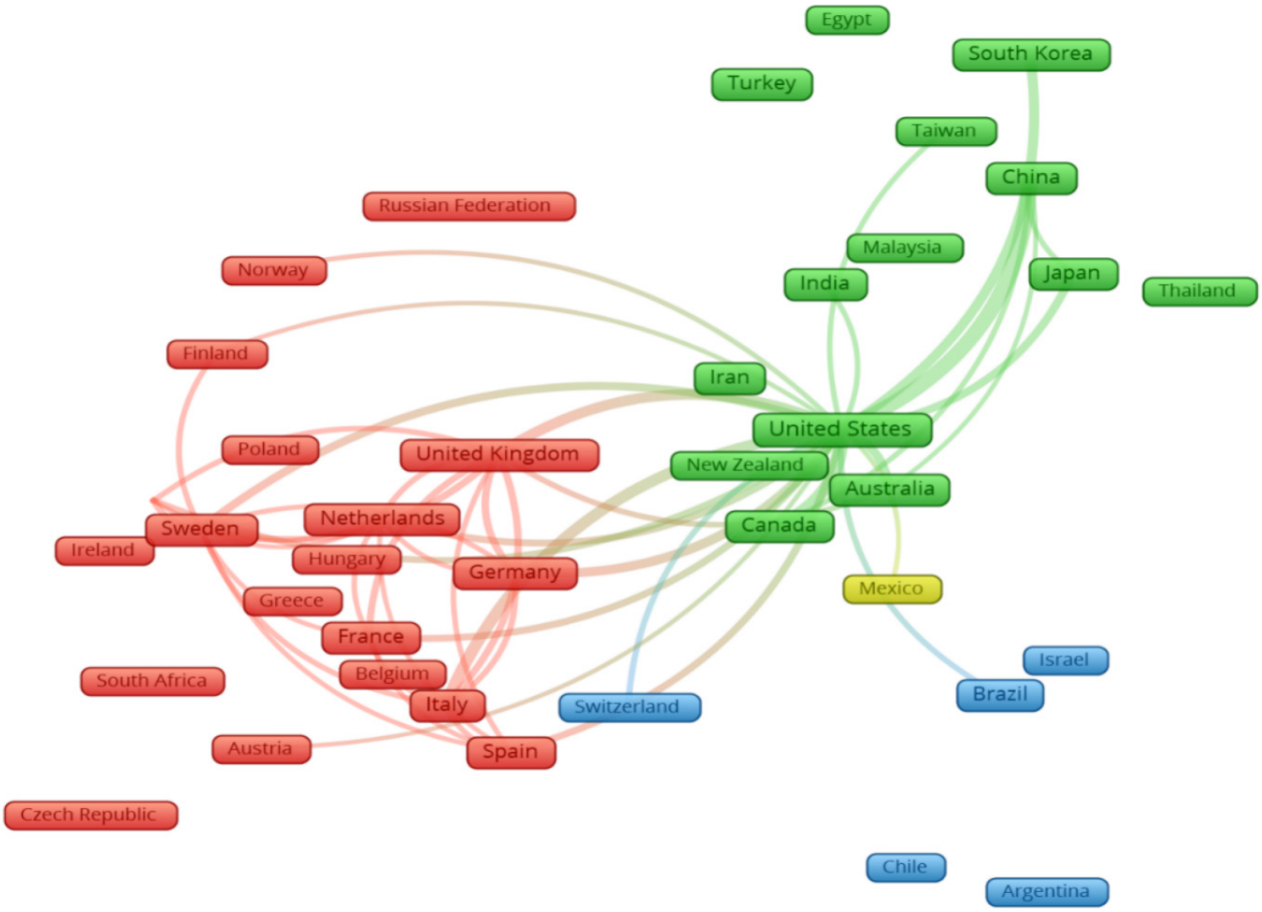
[1]2020 Journal Citation Reports® Science Edition (Clarivate Analytics, 2021).
[2]The Impact Index Per Article is presented based on *Reference Citation Analysis*, https://www.referencecitationanalysis.com [Source: Baishideng Publishing Group Inc (Pleasanton, CA 94566, United States)]. IF: Impact factor.

Table 6 List of top 10 active authors in insulin resistance research, ranked by the total number of publications in the last two decades (2002-2021)

Ranking	Author	Country	*n*	%	*H* index
1st	Shulman GI	United States	150	0.56	154
2nd	Haffner SM	United States	86	0.32	144
3rd	Reaven GM	United States	76	0.28	120
3rd	Roden M	Germany	76	0.28	86
5th	Häring HU	Germany	75	0.28	104
6th	Fritsche A	Germany	70	0.26	80
7th	Fernández-Real JM	Spain	68	0.25	75
7th	Izaola O	Spain	68	0.25	32
7th	Wagenknecht LE	United States	68	0.25	87
10th	Pacini G	Italy	65	0.24	65

subject areas, such as medicine, biochemistry, genetics, and molecular biology, nursing, pharmacology, toxicology, and pharmaceutics, agricultural and biological sciences, neuroscience, and immunology and microbiology journals, revealing the contribution and collaboration of many researchers from different subject areas. Previous research has confirmed that[43-45]. The findings of this study confirm the close association between IF and citations and the fact that the most cited articles are frequently published in journals at the top of the IF list, which helps these journals maintain their high IF.

Furthermore, the increase in insulin resistance publications can be attributed to the fact that numerous hot topics were published during this period[33-37], exposing novel hypotheses and establishing new research fields such as "inflammatory mechanisms in the regulation of insulin resistance" and "mechanisms linking obesity and insulin resistance". Several studies have shown that inflammation is a critical mediator in obesity-induced insulin resistance. Most of these investigations examined the links between adipose tissue in obesity and the regulation of inflammation and insulin resistance[46-49] and the mechanisms by which dietary anti-inflammatory components/functional nutrients may be helpful[50-52].



DOI: 10.4239/wjd.v13.i9.786

Figure 2 Map of visualization of worldwide research collaboration network. Countries with short distances and extensive connecting lines had a significant research collaboration. This collaborative map was built when each country had at least 100 articles. Source: Own elaboration, based on Scopus database; figure created using VOSviewer Software.

Publications with the highest citation frequencies have the greatest academic effect[53,54]. For example, the study published in the Journal of Clinical Investigation in 2003 by Xu *et al*[36] was ranked first. It was revealed that macrophages in white adipose tissue are involved in morbid obesity and that macrophage-associated inflammatory activities may contribute to the pathophysiology of obesity-induced insulin resistance[36]. The article ranked second was published in Diabetes by Cani *et al*[40]. Metabolic endotoxemia was found to alter the inflammatory tone of the body, causing weight gain and diabetes[40].

Strengths and limitations

This is the first bibliometric and visual analysis study to investigate research trends and hotspots in insulin resistance from 2002 to 2021. The current study reviewed linked papers on this issue from numerous perspectives, demonstrated a comprehensive view of understanding in this field during the last few years, and gave direction for future investigations. New researchers in this discipline may simply access meaningful and relevant material with the aid of this bibliometric study. However, certain limitations apply to the generalizability of these findings. First, bibliometric analyses solely used published material from the Scopus database. This may underestimate the amount of research done in South America, China, the Middle East, and other regions of the globe with non-English and unindexed publications. Second, because bibliometric data changes over time, indexing delays may have caused a slight (but not significant) in the number of documents or other metrics. Third, to avoid selection bias, the current study only searched the title for terms such as "insulin resistance" or "insulin sensitivity". As a result, the possibility of false positive or false negative results should always be considered. Fourth, Scopus's results reflect the type and content of Scopus's database. As a result, if prolific authors have two or more Scopus profiles, their research output is likely to be dispersed, and their names may not appear in the active list. The same is true when alternative spellings of an institution's name are used in published documents. As a result, interpreting data about the most active authors, institutions, and nations should be limited to the Scopus findings produced using the described technique.

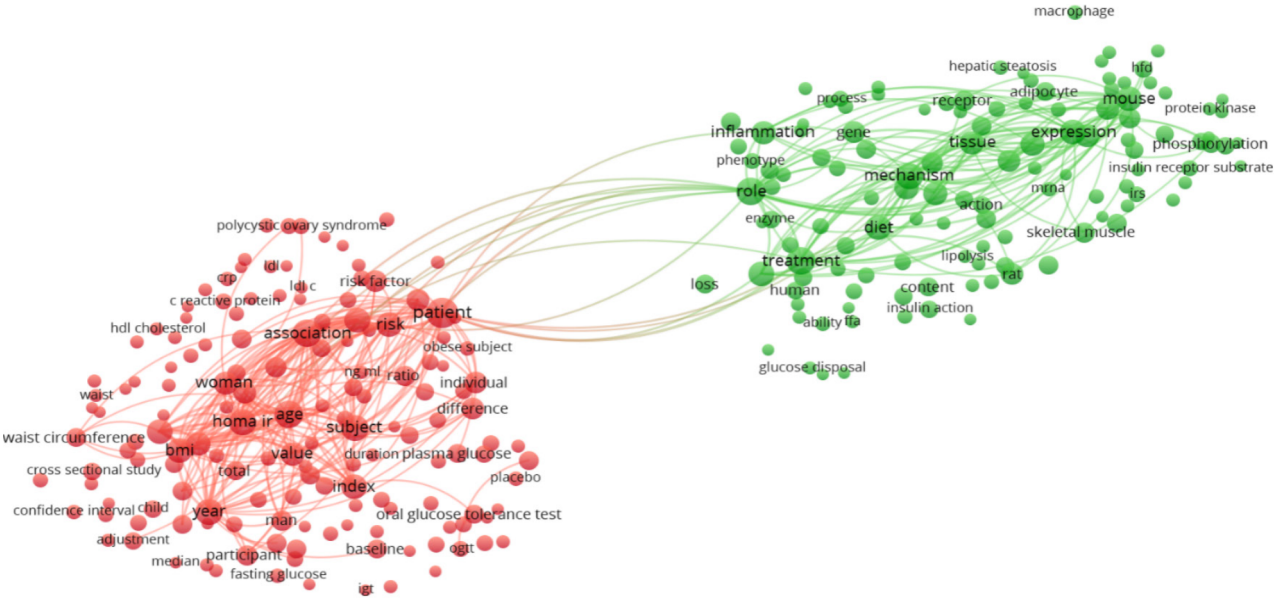


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Figure 3 Network visualization map of terms in the titles/abstracts with a minimum occurrence of 300 or more. Of the 250809 terms in this field, 456 achieved this threshold, were grouped into two clusters, and colored differently. Each cluster represents a general research theme present in the retrieved documents. Source: Own elaboration, based on Scopus database; figure created using VOSviewer Software. LDL-C: Low-density lipoprotein cholesterol; HDL: High-density lipoprotein; IGT: Impaired glucose tolerance; OGTT: Oral glucose tolerance test; BMI: Body mass index; HFD: High fat diet; IRS: Insulin receptor substrate; CRP: C-reactive protein; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance.

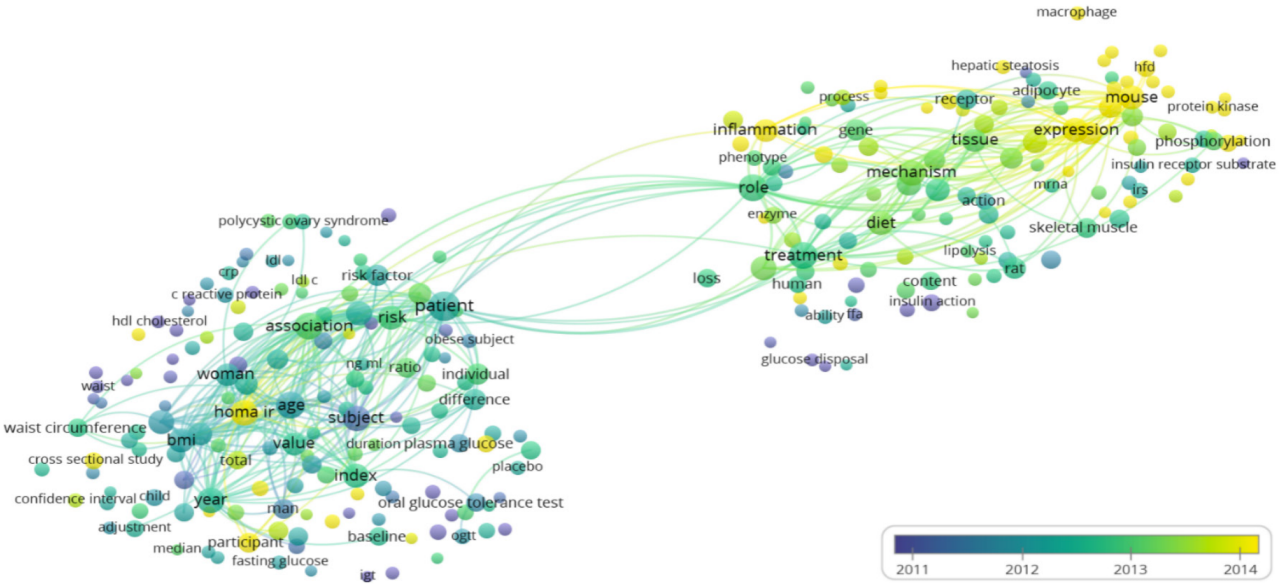

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Figure 4 Network visualization map of terms in the title/abstract according to the average timing of their appearance. Blue represents early appearance, and yellow represents late appearance. Source: Own elaboration, based on Scopus database; figure created using VOSviewer Software. LDL-C: Low-density lipoprotein cholesterol; HDL: High-density lipoprotein; IGT: Impaired glucose tolerance; OGTT: Oral glucose tolerance test; BMI: Body mass index; HFD: High fat diet; IRS: Insulin receptor substrate; CRP: C-reactive protein; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance.

CONCLUSION

To our knowledge, this was the first study to conduct a comprehensive bibliometric analysis of insulin resistance publications from 2002 to 2021, covering the publication year, the number of citations, and current hot topics and trends projected from them. Our data showed that insulin resistance has steadily gained interest from researchers, as evidenced by the number of citations and yearly publications. So far, the United States has been the undisputed leader in this topic, which cannot be divorced from adequate funding sources. Publications have grown significantly in the last decade, while low-income

countries with greater burdens continue to produce fewer publications in this field. "Inflammatory mechanisms in the regulation of insulin resistance" and "mechanisms linking obesity to insulin resistance" were hotspots for insulin resistance research in the past 20 years. This approach might assist researchers in choosing new research areas and recognizing research hotspots and frontiers. In the future, perhaps high-quality clinical evidence will be acquired.

ARTICLE HIGHLIGHTS

Research background

Insulin resistance is a condition in which muscle cells take up and store glucose and triglycerides, resulting in elevated amounts of glucose and triglycerides circulating in the bloodstream.

Research motivation

Several bibliometric studies have been carried out on the subject of diabetic investigation. However, no bibliometric study has been done on research into insulin resistance.

Research objectives

This bibliometric study aimed to identify and assess the current state and trends in insulin resistance research production worldwide and visually analyze research hotspots on this subject.

Research methods

The Scopus database and *Reference Citation Analysis* were used to compile the literature on insulin resistance. In addition, VOSviewer software was used to visually assess data collected from relevant publications.

Research results

This is the first bibliometric analysis of trends in insulin resistance. The number of publications on insulin resistance has increased in the last decade. Our results indicated that the "inflammatory mechanisms in the regulation of insulin resistance" and "mechanisms linking obesity to insulin resistance" will remain research hotspots in the future.

Research conclusions

Our findings indicate that interest in insulin resistance has gradually increased among researchers, as shown by the increasing number of citations and annual publications. Moreover, publications in this field have increased significantly in the last decade, while low-income countries with higher burdens continue to produce fewer publications.

Research perspectives

This paper contributes essential information by providing references and suggestions for future research on pathophysiology and clinical uses of insulin resistance. This approach may aid researchers in identifying new topics of inquiry and identifying research hotspots and frontiers. Perhaps in the future, high-quality clinical evidence will be collected.

FOOTNOTES

Author contributions: Zyoud SH developed the concept for the manuscript, reviewed the literature, designed the study, collected the data, analyzed the data, made significant contributions to the existing literature search and interpretation of the manuscript, and wrote the manuscript; Shakhshir M, Koni A, Abushanab AS, Jairoun AA, Shahwan WM, Al Subu R, Abu Taha A, and Al-Jabi SW participated in interpretation of the data and made revisions to the initial draft; and all authors provided critical review and approved the final manuscript before submission.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: Palestine

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S-Editor: Wang JJ
L-Editor: Filipodia
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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 September 15; 13(9): 799-801

DOI: 10.4239/wjd.v13.i9.799

ISSN 1948-9358 (online)

LETTER TO THE EDITOR

Different nutrient compositions in diet and taking hypoglycemic drugs can modulate gut microbial flora

Zi-Jun Lin, Qiong-Wen Zhang, Xiao-Lan Yu, Bo Zhou, Chang-Wei Liu, Lian-Ping He

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Alkhatib AJ, Jordan; Eseadi C, Nigeria; Xu PF, United States

Received: May 24, 2022
Peer-review started: May 24, 2022
First decision: July 14, 2022
Revised: July 22, 2022
Accepted: August 16, 2022
Article in press: August 16, 2022
Published online: September 15, 2022

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Abstract

The diet structure of diabetic patients is different from that of normal people. Diabetic patients also need to take hypoglycemic drugs to regulate blood sugar. Both dieting and drugs affect the gut microbiota of diabetic patients. In this letter, we discuss that different dietary patterns and the use of hypoglycemic agents may have an impact on changes in gut microbiota in diabetic patients.

Key Words: Diabetic patients; Gut microbiota; Hypoglycemic drugs

Core Tip: Changes in diet can lead to changes in the composition of gut microbiota in diabetic patients. On the other hand, taking hypoglycemic drugs can also change the gut microbiota. Therefore, it is necessary to consider the dietary structure and the use of hypoglycemic drugs in the study of changes in the intestinal flora of patients with diabetes.

Citation: Lin ZJ, Zhang QW, Yu XL, Zhou B, Liu CW, He LP. Different nutrient compositions in diet and taking hypoglycemic drugs can modulate gut microbial flora. *World J Diabetes* 2022; 13(9): 799-801
URL: https://www.wjgnet.com/1948-9358/full/v13/i9/799.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i9.799

TO THE EDITOR

Diabetes mellitus (DM) is one of important risk factor for population health in the twenty-first century worldwide. It is of great significance to explore the lifestyle intervention mode for the prevention and treatment of type 2 diabetes mellitus (T2DM). One study found intermittent hypoxia (IH) was associated with metabolic diseases including as obesity and obstructive sleep apnea-hypopnea syndrome (OSAHS)[1]. IH may be involved in selective alterations of the gut microbiota of T2DM patients with OSAHS. Similarly, changes in gut microbiota can affect the development of T2DM.

Gut microbiota is known to change with diet. Especially when someone suffering from T2DM, doctors often recommend dietary changes to curb the progression of the disease. Changes in eating habits can disrupt the balance of gut microbiota when the body's resistance is low[2]. The study by Liu *et al*[3] showed that blood sugar levels in mouse model of T2DM induced by streptozotocin-high-fat diet were changes with gut microbiota. A large number of studies have shown that diet affects the development of diabetes. For example, blackcurrant extract enhanced insulin sensitivity and glucose-stimulated insulin secretion in non-obese type 2 diabetic rats[4]. Therefore, the diet structure will affect the gut microbiota. However, the following questions need to be further clarified in future studies. What nutrition considerations for persons with diabetes that will impact persons? What are the recommendations for persons with diabetes concerning nutrition to be considered? The link between gut microbial, diabetes, nutrition and autoimmunity are important, but still need to be addressed.

In addition, hypoglycemic drugs also affect gut microbiota. In this study, drugs that regulate IH have an effect on the balance of gut microbiota. We strongly agree with this view. However, when taking drugs to regulate IH, hypoglycemic drugs are also used. Therefore, hypoglycemic drugs also have an impact on the balance of gut microbiota. For some patients who require combination therapy to treat diabetes and complications of diabetes, especially after combination therapy with antibiotics and hypoglycemic drugs, the impact on the intestinal flora is significant[5]. Clinically, metformin is widely used in the treatment of T2DM. Studies have shown that gut microbiota is an active site of metformin. The gut is a potential target of metformin. Metformin induce butyrate and propionate involving glucose homeostasis[6]. When diabetics take metformin, the gut microbiota will definitely change. Studies have also shown that treating diabetic mice with oleuropein (OP) is also treated by modulating the gut microbiota. The OP could decrease fasting blood glucose levels and improve glucose tolerance[7]. Hypoglycemic drugs are also taken when taking drugs that regulate IH, so hypoglycemic drugs will also affect the intestinal flora of patients with IH. It is common for IH patients to take hypoglycemic drugs, so the regulation of hypoglycemic drugs on gut microbiota also needs to be discussed.

On the other hand, IH may be associated with changes in gut microbiota, and it is possible that changes in gut microbiota led to IH. In the clinical setting, the effective treatment for IH is usually oxygen therapy. Thus, we can use oxygen inhalation to intervene in changes in gut microbiota. By conducting oxygen supply experiments, the relationship between IH and gut microbiota can be further reflected, which makes the research results more convincing.

The diet structure of diabetic patients is different from that of normal people. Diabetic patients also need to take hypoglycemic drugs to regulate blood sugar. Both dietary structure of patients with diabetes and hypoglycemic drugs taken by the patients with diabetes can alter the gut microbiota of the patients. Therefore, the influence of diet and drugs on the gut microbiota cannot be ignored.

FOOTNOTES

Author contributions: He LP and Liu CW contributed to the conception of research; Zhang QW, Lin ZJ and Zhou B wrote the manuscript; Zhang QW, Lin ZJ and Yu XL contributed to the revision of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: China

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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https://www.wjgnet.com

ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 October 15; 13(10): 802-899

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

Contents

Monthly Volume 13 Number 10 October 15, 2022

World Journal of Diabetes

Monthly Volume 13 Number 10 October 15, 2022

Contents

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Editorial Board Member of *World Journal of Diabetes*, Debmalya Sanyal, MD, DM, MRCP, FRCP, SCE, FACE, Professor, Department of Endocrinology, KPC Medical College, Jadavpur, Kolkata, West Bengal 700032, India. drdebmalyasanyal@gmail.com

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The primary aim of *World Journal of Diabetes* (*WJD*, *World J Diabetes*) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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INDEXING/ABSTRACTING

The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJD* as 4.560; IF without journal self cites: 4.450; 5-year IF: 5.370; Journal Citation Indicator: 0.62; Ranking: 62 among 146 journals in endocrinology and metabolism; and Quartile category: Q2.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE
October 15, 2022

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World J Diabetes 2022 October 15; 13(10): 802-808

DOI: 10.4239/wjd.v13.i10.802

ISSN 1948-9358 (online)

EDITORIAL

Considerations for management of patients with diabetes mellitus and acute COVID-19

Efterpi Mougakou, Maria Kyziroglou, Alexandra Tsankof, Evangelos Cholongitas, Konstantinos Tziomalos

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Al-Hadhrami R, Oman; Aydin S, Turkey; Liu D, China; Prasad GVR, Canada

Received: March 30, 2022
Peer-review started: March 30, 2022
First decision: May 11, 2022
Revised: May 23, 2022
Accepted: August 17, 2022
Article in press: August 17, 2022
Published online: October 15, 2022

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Abstract

Diabetes mellitus (DM) is an independent risk factor for admission to intensive care unit and death in patients with coronavirus disease 2019 (COVID-19). On the other hand, medications used in the management of COVID-19 are potentially associated with increases in blood glucose levels and a higher incidence of infections. Accordingly, care of patients with DM and acute COVID-19 requires careful consideration of both diseases. Hyperglycemia and hypoglycemia are associated with adverse outcomes and therefore frequent measurement of blood glucose levels and a basal-bolus insulin regimen are required in most patients. Regarding the management of COVID-19, dexamethasone increases blood glucose levels and might also increase the risk for infections. On the other hand, limited data suggest that antiviral and immunomodulatory agents used in COVID-19 are not strongly associated with higher incidence of infections in this population. As knowledge evolves in this field, optimization of the management of both DM and COVID-19 will hopefully improve the outcome of these patients.

Key Words: Diabetes mellitus; COVID-19; Insulin; Antidiabetic agents; Dexamethasone; Tocilizumab; Remdesivir

Core Tip: Diabetes mellitus is a frequent comorbidity in patients hospitalized with coronavirus disease 2019 and is associated with adverse outcomes. Strict glycemic control using insulin is necessary in most of these patients. Dexamethasone, antiviral agents and immunomodulation are also frequently administered and require vigilance and careful monitoring for adverse effects, particularly infections.

Citation: Mougakou E, Kyziroglou M, Tsankof A, Cholongitas E, Tziomalos K. Considerations for management of patients with diabetes mellitus and acute COVID-19. *World J Diabetes* 2022; 13(10): 802-808
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/802.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.802

INTRODUCTION

Several studies showed that diabetes mellitus (DM) is an independent risk factor for contracting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19)[1,2]. Moreover, DM is associated with longer hospitalization, increased risk for admission to an intensive care unit (ICU) and higher mortality in patients with COVID-19[1-3]. Elderly patients and those with poor glycemic control and comorbidities, including hypertension and established cardiovascular disease (CVD), are at higher risk for adverse outcomes[1-3]. In addition, patients with DM and COVID-19 appear to have higher risk for acute complications of DM, particularly diabetic ketoacidosis (DKA)[4]. On the other hand, SARS-CoV-2-induced insulin resistance and impaired insulin production, stress and dexamethasone, which is frequently used for the management of COVID-19, often cause substantial increases in blood glucose levels[5-7]. Furthermore, immunomodulatory agents, which are also part of the treatment of COVID-19, might increase the risk for infection, which is higher in patients with DM[8,9]. Therefore, the management of patients with both DM and COVID-19 requires special considerations, which are briefly summarized in the present commentary.

BLOOD GLUCOSE GOALS

In patients with DM and COVID-19, both hyperglycemia and hypoglycemia have been associated with worse outcome[10,11]. Therefore, maintaining a strict glycemic control in this population appears to be of critical importance. Blood glucose levels between 110 and 180 mg/dL have been recommended as targets in hospitalized diabetic patients with COVID-19, aiming at the higher end of range[12]. However, this target should be individualized, blood glucose levels up to 220 mg/dL are considered acceptable and glucose control should be less strict in patients at high risk for hypoglycemia, including the elderly, the underweight, and patients with severe COVID-19 and/or renal impairment[12].

MONITORING OF BLOOD GLUCOSE LEVELS

Glucose measurement should be performed at least 4 times per day, before meals and at bedtime, but in certain cases has to be done more frequently, particularly in patients who are not eating or are receiving parenteral nutrition[12]. Continuous blood glucose monitoring devices can also be used, particularly in ICU, and appear to be feasible, accurate and reduce the need for point of care glucose measurements [13]. Given the increased risk for DKA in patients with DM and COVID-19, blood ketone levels should ideally be measured in all diabetic patients at admission[12].

ANTIDIABETIC TREATMENT

Regarding antidiabetic treatment, insulin is the agent of choice in most patients. In those who are already receiving long-acting basal insulin, this should be continued[12]. If the patient is not on long-acting insulin and has ≥ 2 blood glucose measurements > 220 mg/dL within the previous day, basal insulin should be started at a total daily dose of 0.25 units/kg[12]. However, in elderly or frail patients and in those with impaired kidney function, the total daily dose of basal insulin should be lower (approximately 0.15 units/kg)[12]. In patients who are receiving glucocorticoids, the dose of basal insulin should be increased by 20%-40%, depending on blood glucose levels[8]. Basal insulin dose are then titrated once-daily according to blood glucose levels, the severity of COVID-19 and caloric intake [12]. Regarding rapid-acting insulin, corrective doses should be administered in patients with blood

glucose levels > 220 mg/dL and the dose should depend on glucose levels and either on total daily dose (in patients who were already using insulin) or on body weight (in patients naïve to insulin)[12]. In critically ill patients and in those who cannot eat, insulin should be administered intravenously[12]. Notably, sliding scale insulin and premixed insulin have been associated with higher risk for iatrogenic hypoglycemia and are not recommended[14]. Patients with type 1 DM can be treated with either subcutaneous or intravenous insulin, depending on their clinical condition. Insulin is administered intravenously at a rate between 1-5 units/h whereas in patients who cannot eat, glucose-dextrose solutions are preferred to avoid hypoglycemia[12]. Regarding patients on insulin pump therapy, this can be maintained provided that their clinical status is stable[12].

Regarding the use of oral antidiabetic agents, metformin should be stopped at admission but if and when the risk of lactic acidosis is considered low, it should be restarted since it appears to improve the outcome of COVID-19[12,15,16]. If used, the dose of metformin should be reduced in patients with estimated glomerular filtration rate (eGFR) between 30 and 45 mL/min and should be discontinued in patients with eGFR < 30 mL/min, liver failure, high risk for lactic acidosis and before iodine contrast imaging[17]. Sulfonylureas are not recommended because of reduced efficacy due to COVD-19-related impaired insulin production and increased insulin resistance and also due to the risk for hypoglycemia, particularly in elderly and in patients with renal impairment or poor oral intake[12]. However, emerging data suggest that these agents might also reduce mortality risk in diabetic patients with COVID-19[18]. Sodium-glucose cotransporter-2 inhibitors should also be discontinued in hospitalized patients, particularly in severely ill patients, due their association with euglycemic DKA[12]. Thiazolidinediones are also not recommended due to their association with edema and heart failure exacerbation, especially in patients with severe COVID-19 and hemodynamic instability[19]. In contrast, dipeptidyl peptidase-4 (DPP-4) inhibitors could be used alone or in combination with insulin in patients with mild hypoglycemia; however, they should be avoided in critically ill patients due to their association with increased risk for heart failure[12]. Notably, some studies suggested that continued use of DPP-4 inhibitors after hospitalization was associated with a decrease in mortality compared with discontinuation but others did not confirm this finding[18,20]. Finally, glucagon-like peptide-1 receptor agonists should also be stopped in hemodynamically unstable and severely ill patients due to risk of gastrointestinal side effects[12].

MANAGEMENT OF COVID-19

Regarding the management of COVID-19, in patients who require supplemental oxygen or ventilatory support, low-dose dexamethasone (6 mg daily for 10 d or until discharge) is recommended, according to data suggesting a clear benefit on all-cause 28-day mortality[21-23]. Indeed, in the controlled, open-label RECOVERY trial (n = 2104 patients assigned to receive dexamethasone and 4321 to receive usual care), the 28-day mortality was 36% lower in the dexamethasone group among patients on mechanical ventilation and 18% lower among those on supplemental oxygen[23]. Of note, 24% of the total study population had DM and no excess serious adverse events related to dexamethasone were recorded[23]. The incidence of death due to infections other than COVID-19 also did not differ between patients treated with dexamethasone and those assigned to usual care[23]. According to a meta-analysis by the World Health Organization Rapid Evidence Appraisal for COVID-19 Therapies Working Group, which included 7 trials in 1703 critically ill patients with COVID-19, administration of glucocorticoids was associated with 34% lower 28-d mortality with no suggestion of a higher risk of adverse effects compared with standard of care or placebo[22]. Despite these reassuring findings, patients with diabetes receiving glucocorticoids should be carefully monitored for bacterial or fungal infections, with prompt initiation of empirical antibiotic treatment if needed[8].

In patients with COVID-19 who require supplemental oxygen, but not in those on mechanical ventilation or extracorporeal membrane oxygenation, the antiviral agent remdesivir (200 mg intravenously on day 1 followed by 100 mg/d for 5 d) should be considered because it shortens recovery time and shows a trend for reduced need for mechanical ventilation and improved survival[21,24,25]. In a trial in 1062 patients hospitalized with COVID-19 pneumonia randomized to receive remdesivir or placebo (30.6% with DM), hyperglycemia was a common non-serious adverse effect, occurring in 6% of patients, but with a similar incidence in the remdesivir and the placebo group[24]. The rate of infections was also similar in the 2 groups[24]. Remdesivir can also be considered in hospitalized patients without requirement for supplemental oxygen. In a randomized, open-label trial (n = 584 patients with moderate COVID-19, defined as any radiographic evidence of pulmonary infiltrates and oxygen saturation > 94% on room air), clinical status at day 11 was better in patients randomized to a 5-d course of remdesivir compared with standard care whereas the incidence of adverse events was similar in the 2 groups[26]. Of note, 40% of patients enrolled in this trial had DM but it was not evaluated whether the benefits and risks of remdesivir differed between this subgroup and non-diabetic patients[26].

Immunomodulatory agents can also be considered in diabetic patients who are hospitalized due to COVID-19. Tocilizumab, an interleukin-6 inhibitor (8 mg/kg as a single intravenous dose), may be used

Table 1 Principles of the management of patients with diabetes mellitus and acute coronavirus disease 2019

	Principles of the management
Blood glucose goals	Between 110 and 180 mg/dL in most patients. Less strict goals in patients at high risk for hypoglycemia
Monitoring of blood glucose levels	At least 4 times daily. More frequently in selected patients (*e.g.*, in the intensive care unit)
Antidiabetic treatment	Insulin in most patients. Metformin and dipeptidyl peptidase-4 inhibitors might be considered. Other antidiabetic agents should be avoided
Management of COVID-19 in hospitalized patients	Similar to non-diabetic patients. Patients receiving glucocorticoids or immunomodulatory agents should be carefully monitored for infections
Management of COVID-19 in the outpatient setting	Patients with symptomatic COVID-19 are eligible for treatment with monoclonal antibodies, remdesivir, nirmatrelvir-ritonavir or molnupiravir

COVID-19: Coronavirus disease 2019.

in patients who require high-flow oxygen or mechanical ventilation and it may also be an option for selected patients on low-flow oxygen with significantly elevated inflammatory markers (C-reactive protein levels ≥ 75 mg/L) or with a rapid increase in oxygen requirements despite dexamethasone therapy, within 96 h of hospitalization[25]. In a meta-analysis of 10930 patients hospitalized for COVID-19, administration of tocilizumab was associated with a 17% lower all-cause 28-d mortality with no increased risk of infection compared with standard of care or placebo[27]. Sarilumab may be an alternative interleukin-6 inhibitor option if tocilizumab is not available, but with limited trial data[27]. Another treatment option is baricitinib (4 mg/day orally for 14 d), a Janus Kinase inhibitor with immunomodulatory properties, that may be used with the same indications as tocilizumab, with the exception of patients on mechanical ventilation due to limited trial data in this subgroup of patients[25]. In a randomized, placebo-controlled trial in 1525 patients (30% had DM), baricitinib reduced 28-d and 60-d mortality by 38% without an increased risk for infection or other adverse events[28]. Notably, it has not been evaluated whether these immunomodulatory agents have different safety or efficacy in patients with DM[27,28].

Notably, patients with acute COVID-19 are at higher risk for thrombosis than the general inpatient population and the presence of DM further increases this risk[29]. Accordingly, this population should be carefully monitored for the occurrence of thrombotic events and should receive prophylactic dose of heparin[21,25]. In patients who are already receiving antiplatelet agents for DM or for established CVD, these should be continued and low-dose heparin should be added[21,25].

Regarding the outpatient management of COVID-19, even in the absence of symptoms of severe disease, both patients with type 1 and 2 DM are considered at high risk for evolution to severe disease, especially if they are ≥ 65 years-old or have obesity, chronic kidney disease or established CVD[30]. Therefore, patients with DM and symptomatic COVID-19 are eligible for treatment with monoclonal antibodies, remdesivir, nirmatrelvir-ritonavir or molnupiravir, to reduce the risk of hospitalization[21,25]. The choice between these agents depends mainly on availability and should start as soon as possible after symptom onset[21,25]. There is no specific agent that is contraindicated in patients with DM, however nirmatrelvir-ritonavir cannot be used if eGFR is < 30 mL/min[21,25]. Moreover, before prescription of ritonavir-boosted nirmatrelvir, a careful review of concomitant medications is required, because it has significant drug-drug interactions with commonly prescribed medications in patients with DM and CVD, including rosuvastatin, clopidogrel and rivaroxaban[21,25].

Patients with DM also appear to be at higher risk for persistence of COVID-19-related symptoms (*i.e.*, long COVID)[31]. It has also been reported that an aggravation of insulin resistance persists for up to 2 mo after recovery from COVID-19[32]. Accordingly, patients with DM should be followed up closely after the resolution of COVID-19.

CONCLUSION

Diabetic patients with acute COVID-19 are a particularly vulnerable population at a high risk for complications. Close monitoring of blood glucose levels and careful administration of insulin with appropriate titration are needed to achieve glycemic control without complications. On the other hand, management of COVID-19 in these patients requires individualization and heightened attention for the occurrence of adverse events, particularly hyperglycemia and infections (Table 1). There are currently limited data regarding the safety and efficacy of both antidiabetic and antiviral treatments in diabetic patients with acute COVID-19. As knowledge evolves in this field, optimization of the management of both DM and COVID-19 will hopefully improve the outcome of these patients.

FOOTNOTES

Author contributions: Mougakou E, Kyziroglou M and Tsankof A drafted the manuscript; Cholongitas E and Tziomalos K critically revised the draft.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Gao CC
L-Editor: A
P-Editor: Gao CC

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World Journal of Diabetes

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World J Diabetes 2022 October 15; 13(10): 809-821

DOI: 10.4239/wjd.v13.i10.809

ISSN 1948-9358 (online)

REVIEW

Growing importance of urogenital candidiasis in individuals with diabetes: A narrative review

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Specialty type: Mycology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Rwegerera GM, Botswana; Tu WJ, China

Received: May 13, 2022
Peer-review started: May 13, 2022
First decision: August 1, 2022
Revised: August 6, 2022
Accepted: September 8, 2022
Article in press: September 8, 2022
Published online: October 15, 2022

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Abstract

Both diabetes and fungal infections contribute significantly to the global disease burden, with increasing trends seen in most developed and developing countries during recent decades. This is reflected in urogenital infections caused by *Candida* species that are becoming ever more pervasive in diabetic patients, particularly those that present with unsatisfactory glycemic control. In addition, a relatively new group of anti-hyperglycemic drugs, known as sodium glucose cotransporter 2 inhibitors, has been linked with an increased risk for colonization of the urogenital region with *Candida* spp., which can subsequently lead to an infectious process. In this review paper, we have highlighted notable virulence factors of *Candida* species (with an emphasis on *Candida albicans*) and shown how the interplay of many pathophysiological factors can give rise to vulvovaginal candidiasis, potentially complicated with recurrences and dire pregnancy outcomes. We have also addressed an increased risk of candiduria and urinary tract infections caused by species of *Candida* in females and males with diabetes, further highlighting possible complications such as emphysematous cystitis as well as the risk for the development of balanitis and balanoposthitis in (primarily uncircumcised) males. With a steadily increasing global burden of diabetes, urogenital mycotic infections will undoubtedly become more prevalent in the future; hence, there is a need for an evidence-based approach from both clinical and public health perspectives.

Key Words: Balanitis; Balanoposthitis; Candida; Candidiasis; Diabetes; Pregnancy; Urogenital infections; Vulvovaginitis

Core Tip: The global health burden of both diabetes and *Candida* spp. infections is on the rise, and these two clinical entities can have a compounding effect on the development of different urogenital diseases and syndromes. Pathophysiological changes observed in diabetes mellitus can predispose individuals to *Candida* colonization, increased virulence of this fungus, and subsequent infection. Diabetic females are more prone to recurrent vulvovaginal candidiasis that can endanger the pregnancy, while diabetic males have higher rates of balanitis/balanoposthitis. In both females and males, there is an increased risk of candiduria and urinary tract infections, with complications such as emphysematous cystitis.

Citation: Talapko J, Meštrović T, Škrlec I. Growing importance of urogenital candidiasis in individuals with diabetes: A narrative review. *World J Diabetes* 2022; 13(10): 809-821
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/809.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.809

INTRODUCTION

Diabetes is a salient global health issue, with an enormous disease burden that has increased substantially in recent decades for the majority of developed and developing countries. The estimations from the International Diabetes Federation reveal that 537 million adults are living with diabetes around the world, with a projected growth to 693 million or more by 2045 without effective preventative methods [1,2]. On the other hand, the estimates from the Global Action Fund for Fungal Infections show that every year there are over 300 million individuals of all ages suffering from a fungal infection that can seriously impact their health[3], which also includes urogenital infections caused by yeasts belonging to the genus *Candida*.

Taking into account such considerable global prevalence of these two frequently coexistent clinical conditions, it is of no wonder that diabetic patients with genitourinary candidiasis are currently pervasive not only in primary practice but also in secondary and tertiary care facilities. In addition, a relatively new group of anti-hyperglycemic drugs known as sodium glucose cotransporter 2 (SGLT2) inhibitors made both females and males more prone to *Candida* colonization of the urogenital region as well as for subsequent infection[4-6]. All of this means urogenital *Candida* infections may become even more ubiquitous among diabetic patients in the future. Therefore, given the scarcity of recent and comprehensive sources that provide an integrative and critical overview of the available literature on this topic (Table 1), in this review we aimed to summarize microbiological, pathophysiological, and clinical facets of urogenital infections with *Candida* species in both females and males with diabetes.

DIABETES AND IMMUNE RESPONSE AGAINST INFECTIONS

According to the classification published by the American Diabetes Association, diabetes occurs in four basic forms, of which diabetes mellitus type 1 and diabetes mellitus type 2 are the most common forms of the disease[7]. In the quotidian clinical approach, fasting blood glucose levels up to 5.6 mmol/L are normal. When these values are above 7 mmol/L, this represents a key criterion for diagnosing diabetes mellitus, while values between 5.6 mmol/L and 6.9 mmol/L indicate prediabetes[8]. Therefore, it is always necessary to perform two glucose measurements: the first one on an empty stomach; and the second 1-2 h after a meal. Glucose values 2 h after a meal should fall below 7.8 mmol/L; if these values are still above 11 mmol/L, then we can diagnose diabetes mellitus with a substantial amount of certainty. If these values are between 7.8 to 11 mmol/L, we consider prediabetes or glucose intolerance [9]. The vital difference between prediabetes and diabetes is that prediabetes can be reversed. Of course, the most crucial factors are lifestyle changes, but there are also several viable pharmacological approaches.

Type 1 diabetes mellitus is caused by an absolute (or almost absolute) lack of insulin due to autoimmune destruction of pancreatic β-cells, which leads to insulin insufficiency and hyperglycemia [10]. Conversely, type 2 diabetes mellitus is characterized by insulin resistance with an inadequately compensatory increase in insulin secretion[11]. Gestational diabetes occurs in pregnancy, most often during the second trimester of pregnancy. Insulin resistance is potentiated by hormones produced by

Table 1 Keywords, database, and search time

Keywords	MeSH term	Database
Balanitis	Balanitis	PubMed, Scopus, RCA
Balanoposthitis	-	PubMed, Scopus, RCA
Vulvovaginitis	Vulvovaginitis	PubMed, Scopus, RCA
Urogenital infections	Urogenital system; infections; pathogenicity	PubMed, Scopus, RCA
Pregnancy	Pregnancy	PubMed, Scopus, RCA
Candida	Candida	PubMed, Scopus, RCA
Candidiasis	Candidiasis	PubMed, Scopus, RCA
Diabetes	Diabetes mellitus; diabetes insipidus	PubMed, Scopus, RCA

RCA: Reference Citation Analysis.

the placenta[12]; therefore, it occurs in females whose pancreatic function does not overcome pregnancy-related insulin resistance. The main consequences are increased risks of preeclampsia, macrosomia, as well as Cesarean delivery and their associated morbidities[13].

Diabetes mellitus is one of the most common endocrine disorders characterized by a disorder in insulin secretion and its action. Due to its frequency, it is currently a global health problem[14]. The prevalence of diabetes mellitus is constantly increasing in developed and developing countries alike. According to the data from 2017, its prevalence is around 8.8% worldwide[15]. In addition to a myriad of co-occurring problems characteristic of patients with diabetes mellitus, a particular issue is immune system dysfunction resulting from complex interactions between the endocrine and immune systems [16]. Immune dysfunction occurs due to elevated insulin levels (hyperglycemia) and leptin present in affected individuals, resulting in an increased risk of various organ damage[17].

Decreased immunity is manifested in decreased T lymphocyte count, reduced cytokine release, increased programmed leukocyte cell death, reduced neutrophil function, impaired ability to fight infectious agents, and increased susceptibility to infection[14]. The increased risk of opportunistic infections is a particular problem due to the weakened ability to fight invasive pathogens[18]. In patients with diabetes mellitus, the recovery time after infection is significantly prolonged compared to individuals without it[19]. One of the salient indicators that should raise a suspicion of underlying diabetes is a propensity for recurrent infections caused by opportunistic pathogenic fungal species belonging to the genus *Candida*[20]. The pathogenic abilities of *Candida* species and their colonization factors depend on host-related immune factors due to the intricate homeostatic relationship of fungi with the host's current immune status, a key determinant of commensalism or parasitism[21]. From a pathophysiological perspective, we find a suitable environment in diabetic patients for *Candida* multiplication and proliferation due to alteration of gut microbiota, dietary changes, reduced intestinal secretions and altered liver function, continued usage of antimicrobial agents (and other drugs), coexisting diseases, as well as the pervasive deficiency of key nutrients, as demonstrated in the literature [21].

CANDIDA AS A PARAMOUNT FUNGAL PATHOGEN

The profile of Candida albicans and non-albicans Candida species

Fungal infections caused by *Candida* species lead to a significant health burden, causing high mortality rates, hospitalizations, and increased treatment costs[22]. Lethal outcomes are most commonly seen as a result of sepsis and invasive systemic candidiasis[23].

Candida albicans was the most widespread fungal pathogen isolated during episodes of candidiasis for a long time. Still, recent literature reports reveal an increasingly important role of other non-*albicans* species such as *Candida glabrata* (*C. glabrata*), *Candida parapsilosis* (*C. parapsilosis*), *Candida krusei* (*C. krusei*), *Candida tropicalis* (*C. tropicalis*), and more recently *Candida auris* (*C. auris*)[24]. However, the most commonly isolated *Candida* spp. from clinical specimens are non-*albicans* species. These other non-*albicans Candida* species are becoming more noticeable due to the production of virulence factors that were once attributed exclusively to *C. albicans*; furthermore, they are also characterized by reduced sensitivity to the most commonly used antifungal drugs[25]. The prevalence and virulence of non-*albicans Candida* species show varied geographical distribution, but more importantly many non-*albicans Candida* species cause more frequent fungal infections in patients with diabetes. That is especially pertinent for patients with type 1 and 2 diabetes mellitus with foot ulcers and skin and nail lesions[6].

Considering all of the above, species-level identification of *Candida* spp. should be introduced into routine laboratory work-up[26].

But notwithstanding such global prominence of non-*albicans* candida, *C. albicans* is still the most common cause of candidiasis[27]. It can be a colonizer of skin and many mucosal surfaces and can thus easily act as an opportunistic pathogen in the genitourinary system[28]. Approximately 75% of females have at least one episode of vulvovaginal candidiasis during their lifetime, and the most common cause (*i.e.* in 90% of cases) the putative species is *C. albicans*[29]. According to available data, in females with diabetes mellitus who presented with a vulvovaginal infection caused by *Candida*, *C. albicans* is the most prevalent fungus in over 50% of cases, while different non-*albicans Candida* species are present in about 40% of cases[30].

Candida is a polymorphic fungus that, contingent on the environment in which it is located, can alter its morphology from yeast form (blastoconidia) to pseudohyphae and hyphae[31]. Indeed, this is one of the most important differences from other *Candida* species because it can create true hyphae *in vivo* when met with favorable conditions[32]. Two serotypes of *C. albicans* have been identified, namely type A and type B[33], and numerous factors contribute to the noticeable increase in invasive fungal infections, including hyperglycemia[19].

Major virulence factors in C. albicans

Virulence represents the ability of a microorganism to damage a host[34], and *C. albicans* possesses a panoply of virulence factors[35]. One of the most important factors is dimorphism (already mentioned), which represents the ability of *C. albicans* to change its shape from yeast to mold, with subsequent formation of true hyphae under favorable conditions. The latter trait significantly increases its invasiveness and proteolytic activity; however, in yeast form, it shows the propensity for greater dissemination[36]. Genes that are important for these activities are *ALS3*, *SAP4-6*, *HWP1*, *HYR1*, and *ECE1*, and their expression can be variable[37], while *SAP1* and *SAP3* and *SAP8* genes have been correlated with vaginal infections[38].

In the first phase of the infection, which is the adhesion phase, adhesins and invasins allow *C. albicans* cells to adhere to the substrate, forming a basal layer of cells[39]. Adhesins are glycoproteins that enable yeast to adhere to epithelial and endothelial cells[40]. Invasins are specialized proteins by which *C. albicans* stimulates host cells towards endocytosis by binding to host cell ligands[41]. The target ligands are E-cadherin on epithelial cells and N-cadherin on endothelial cells[42]. Numerous genes are involved in adhesion to epithelial cells, and the large cell surface area of the glycoprotein encodes eight genes belonging to the *C. albicans* agglutinin-like sequence family[43].

Biofilm production is recognized as a crucial virulence factor (Table 2)[44]. In the proliferation stage of *C. albicans* cells, filaments are formed, in which yeast cells begin to develop filamentous hyphae. That is the most critical step in which cells can change their morphology, facilitating in turn biofilm formation on the mucosal surfaces of the host[45,46]. The biofilm formation process is controlled by six genes (*EFG1, BCR1, BRG1, NDT80, TEC1,* and *ROB1*) that belong to the transcriptional regulatory network[47,48].

Alongside the aforementioned virulence factors, it is also becoming clear that *C. albicans* isolated from patients with diabetes mellitus has more pronounced pathogenic properties[49]. Namely, the hyperglycemic environment, rich in carbohydrates, serves as a source of energy indispensable for producing biofilms and matrices that protect fungal cells from external influences[6]. Most pathological conditions caused by *C. albicans* are associated with biofilm formation on abiotic surfaces or host surfaces[50]. Yeast cells dispersed from mature biofilm are more virulent and have a more remarkable ability to adhere to surfaces to form new biofilms than planktonic ones[51]. Biofilm production also complicates treatment and contributes to high morbidity and mortality rates[52].

C. albicans can produce the cytolytic enzyme known as candidalizine, and hyphae are responsible for its secretion[44]. This enzyme plays a vital role in developing vaginal mucosal infections[53]. More specifically, candidalizine has immunomodulatory properties critical in host cell damage[54] and plays a role in neutrophil recruitment during disseminated systemic fungal infections[55].

A direct contribution to the virulence of *C. albicans* is the secretion of hydrolytic enzymes aspartyl proteinase and phospholipase as well as hemolysin, which all enhance pathogenic effects such as binding to host tissue and rupture of the cell membrane. As a result of their activity, the invasion of the mucosal surface is facilitated, and they are also responsible for avoiding the host's immune response[46,56,57]. In *C. albicans*, at least ten members of the aspartyl proteinase gene family are present, while phospholipase has been reported in four families[58].

Finally, one of the essential contributors to *C. albicans* virulence is thigmotropism (contact sensing), which is regulated by extracellular calcium intake and aids significantly in spreading into host tissues and biofilm development[44].

Table 2 Biofilm production process

Phase	Phase name	Description
1	Adherence	In the first 3 h, individual *C. albicans* cells adhere to the substrate, which forms the basal layer of the biofilm
2	Intermediate phase	In 11-14 h, biofilm is shaped during this phase of cell proliferation and filamentation, in which the formation of hyphae occurs, marking the beginning of true biofilm formation
3	Maturation phase	In 20-48 h, there is a complete penetration of all layers of cells attached to the surface; extracellular polysaccharide matrix accumulates at this stage of maturation
4	Dispersion	After 24 h, the final phase involves separation of non-adherent cells from the biofilm, resulting in possible development of new biofilms and dissemination in the tissue

C. albicans: *Candida albicans*.

TYPES OF UROGENITAL CANDIDIASIS IN PATIENTS WITH DIABETES

Vulvovaginal candidiasis in females with diabetes

Several important pathophysiological mechanisms are involved in the occurrence of vulvovaginitis and vulvovaginal candidiasis (VVC) in individuals with uncontrolled hyperglycemia, leading to increased glucose levels in vaginal mucosa[6]. First of all, yeasts can utilize the glucose found in secretions as a viable nutrient, and additional influence of the overall change in pH and temperature can result in increased *Candida* spp. virulence[59]. Furthermore, the binding of *Candida* spp. to epithelial cells on the vaginal surface represents a pivotal initial step in colonization and ensuing infection with yeasts[60], with an indispensable role of intercellular adhesion molecule 1 expression for facilitating adhesion after the episodes of hyperglycemia[61]. Recurrent episodes of VVC are more frequent in diabetic patients due to immune suppression, altered leukocyte function, and a myriad of other factors[21].

Several different author groups appraised the association between VVC and diabetes mellitus. For example, Gunther *et al*[30] studied females with diabetes from Brazil and found that *Candida* species were more frequently isolated in them than in those without it (18.8% *vs* 11.8%); likewise, the development of VVC (both isolated and recurrent forms) has been more frequently observed in the diabetic group of patients, together with lower cure rates. In a study on postmenopausal females with diabetes and symptoms of VVC, *Candida* spp. were isolated in 15.6% of involved patients using culturing techniques and molecular confirmation with *C. albicans* leading the way in frequency (59.30%), followed by C. *glabrata* (24.41%) and *C. krusei* (16.27%)[62]. These studies also showed different antifungal susceptibilities of isolated species, which is why mycological culture is often endorsed, even though microscopy is often sufficient for visualizing recognizable fungal elements such as pseudohyphae of *C. albicans* (Figure 1).

However, non-*albicans Candida* species are increasingly implicated in VVC in cases of patients with diabetes. In a research endeavor by Ray *et al*[63], which explored cure rates of different treatment modalities, *C. glabrata* has been cultured in 61.3% and *C. albicans* in 28.8% of 111 female individuals with VVC and diabetes. A study by Goswami *et al*[64], conducted on diabetic females from India, showed a relatively high prevalence (46%) of VVC with a relative risk of 2.45 and a predominance of *C. glabrata* and *C. tropicalis*. Such dominance of *C. glabrata* in the same context was confirmed by another study from India[65], showing that all therapeutic considerations have to consider country- and region-specific pathogen distribution (Table 3).

The problem is further aggravated with the use of relatively novel hypoglycemic agents that are known to induce glycosuria, and this specifically pertains to SGLT2 inhibitors. More specifically, the colonization rate with *Candida* spp. (and subsequently the risk of VVC) can increase substantially with the use of these agents, reaching up to 37%[4,5]. Another important issue is selecting the optimal treatment approach in females with recurrent VVC and diabetes, especially since many author groups recommend routine prophylactic administration of antimicrobial drugs in preventing candidiasis when faced with uncontrolled diabetes[6,21]. The best approach is still a matter of debate, as even a recent and comprehensive Cochrane review on different pharmacological and non-pharmacological treatment modalities highlighted that more research is necessary to ascertain the optimal medication choices as well as dose and frequency for females with diabetes[66].

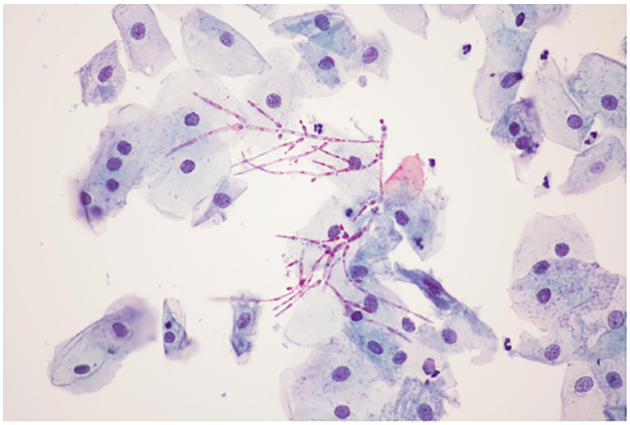
Balanitis/balanoposthitis due to Candida spp. in males with diabetes

The influence of diabetes on the development of balanitis/balanoposthitis caused by *Candida* spp. is well known due to the well-established association of uncontrolled blood glucose levels and the proliferation of *Candida* beneath the prepuce[67]. While *Candida* is a causative agent of less than 20% of all balanoposthitis cases, it is the most commonly observed pathogen in males with diabetes, habitually presenting as a pruritic rash with sores, erosions, or papules (with possible sub-preputial discharge)[68]. In addition, coinfection with other pathogens can worsen the clinical presentation in males with

Table 3 Studies of the prevalence of candidiasis in individuals with diabetics

Ref.	Yr	Study population	Study outcome
Goswami *et al* [64]	2000	*n* = 78 diabetics, *n* = 88 non-diabetics	A total of 46% of diabetic patients showed vaginal *Candida* sp. and 23% healthy subjects demonstrated *Candida* spp.
Goswami *et al* [65]	2006	*n* = 85 diabetics, *n* = 62 non-diabetics	A total of 67.1% of diabetic patients showed vaginal *Candida* spp. and 47.3% healthy subjects demonstrated *Candida* spp. following fluconazole treatment
Gunther *et al*[30]	2014	*n* = 48 diabetics; *n* = 669 non-diabetics	A total of 18.8% of diabetics showed vaginal *Candida* spp. and 11.8% healthy subjects demonstrated *Candida* spp.
Yokoyama *et al* [5]	2019	65 diabetic patients	A total of 36.9% of diabetic patients converted to a positive vaginal *Candida* spp.
Halteet *et al*[62]	2020	550 diabetic patients	A total of 15.6% of diabetics showed vaginal *Candida* spp.
Lisboa *et al*[71]	2010	*n* = 38 diabetics; *n* = 440 non-diabetics	A total of 26.2% of males had *Candida* spp. and 18% of males had balanitis; 13.8% of diabetic patients had balanitis
Kofteridis *et al* [84]	2009	*n* = 88 diabetics; *n* = 118 non-diabetics	A total of 12.7% of diabetic patients showed urinary tract *Candida* spp. and 1.7% healthy subjects demonstrated *Candida* spp.
Yismaw *et al*[90]	2013	422 diabetic patients; *n* = 387 with asymptomatic UTI; *n* = 35 with symptomatic UTI	A total of 17.1% of symptomatic diabetic patients showed significant candiduria and 7.5% of asymptomatic diabetic patients
Falahati *et al*[91]	2016	305 diabetic patients	A total of 12.5% of diabetic patients were positive for candiduria
Esmailzadeh *et al*[89]	2018	400 diabetic patients	A total of 10% of diabetic patients showed *Candida* spp. in the urinary tract
Gharanfoli *et al* [92]	2019	500 patients with UTI; *n* = 106 diabetics; *n* = 394 non-diabetics	A total of 21.1% of diabetic patients showed *Candida* sp. in urinary tract and 4.2% of UTI patients were positive for *Candida* spp.

UTI: Urinary tract infections.



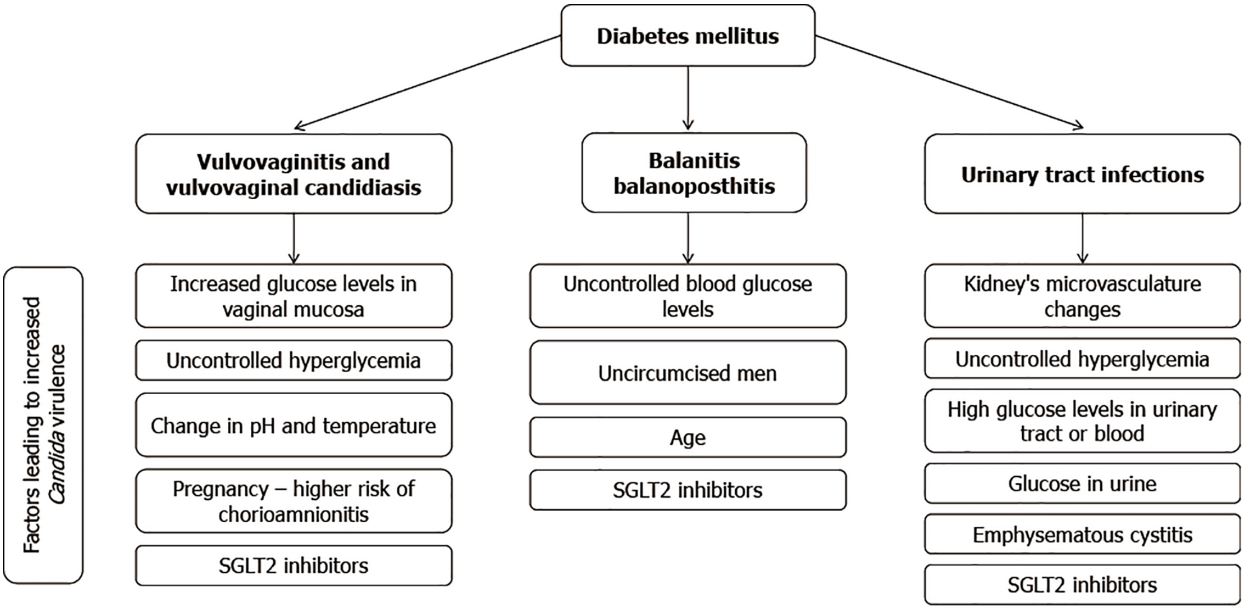
DOI: 10.4239/wjd.v13.i10.809

Figure 1 Filamentous growth of *Candida albicans* in a vaginal specimen, with visible pseudohyphae and hyphae (magnification × 400).

diabetes (not only with common sexually transmitted infections but also pathogens such as *Streptococcus pyogenes*[69]).

The aforementioned connection between diabetes and penile infection is reflected in population studies as well; for example, the appraisal of all male patients with balanoposthitis from the Longitudinal Health Insurance Database in Taiwan revealed that the incidence of type 2 diabetes mellitus was higher in the balanoposthitis cohort than those without it, with a hazard ratio of 2.55 after age and comorbidity adjustments[70]. Furthermore, a large study from Portugal demonstrated that diabetes mellitus was significantly more prevalent in patients with clinically frank balanitis when compared to the asymptomatic group, and there was also higher colonization with *Candida* species[71]. In addition, an extensive survey of dermatology specialists from across India, with more than 60000 outpatients in their care, showed that up to 75% of individuals with *Candida* balanoposthitis were known cases of diabetes mellitus[72].

Even novel hypoglycemic agents that can induce glycosuria, most notably already mentioned SGLT2 inhibitors, can also increase the risk of genital candidiasis in males (Figure 2). For example, a recent



DOI: 10.4239/wjd.v13.i10.809

Figure 2 A summary of factors leading to urogenital candidiasis in patients with diabetes mellitus. SGLT2: Sodium glucose cotransporter 2.

report by Bartolo *et al*[73] showed the development of balanitis due to *C. albicans* and subsequent candidemia but also the potential role of other species such as *C. glabrata*. A severe form of balanoposthitis caused by *C. albicans* after treatment with SGLT2 was also described in a 57-year-old with type 2 diabetes coupled with oral candidiasis[74]. Of note, balanitis is rarely seen in circumcised males, as the moist space beneath the foreskin represents an ideal environment for facilitated yeast proliferation[4].

The compounding effect of diabetes and genital candidiasis in pregnancy

The observed incidence of VVC during pregnancy is approximately 15%[75], but this percentage is even higher in pregnant females with either type 1/type 2 diabetes mellitus or gestational diabetes[76]. This means both pregnancy as a physiological process and diabetes as a pathological condition may have a compounding effect in the development of VVC (Figure 2). In a study on 251 pregnant females from Poland, Nowakowska *et al*[77] demonstrated a four times increased risk of developing vaginal mycosis in those with type 1 diabetes mellitus as well as a two times increased risk in those with gestational diabetes in comparison with healthy controls. A study on pregnant females from a Malaysian tertiary-care hospital showed that the first and second trimester of pregnancy and diabetes mellitus are significant risk factors for developing VVC[78]. A prospective study from China showed a significantly higher frequency of VVC in females with gestational diabetes (22.6% *vs* 9.7%)[76].

This is important due to the possible development of candida chorioamnionitis in diabetic pregnant females stemming from VVC, with potentially detrimental consequences for the unborn child. Although this clinical entity is relatively uncommon, it was repeatedly described in the medical literature. One of the gravest examples is a case reported by Obermair *et al*[79] on *Candida* chorioamnionitis that successively led to a late stillbirth in a pregnant woman with gestational diabetes mellitus. Unfortunately, there were no prior obstetrics procedures in this case, and infection with *C. albicans* triggered an inflammatory cascade that resulted in the occlusion of umbilical cord blood vessels, ultimately resulting in fetal death[79].

Recently, Shazniza Shaaya *et al*[80] reported 2 cases of *Candida* chorioamnionitis linked to gestational diabetes and originating from VVC where manifold red and yellowish spots were observed during pathohistological observation on the superficial area of the umbilical cord. Microscopically, these spots were microabscesses laden with yeasts and pseudohyphae, while peripheral funisitis was highlighted as a prominent feature of such *Candida* chorioamnionitis. Other reported cases of *Candida* chorioamnionitis associated with diabetes mellitus also led to adverse perinatal outcomes such as preterm birth, neonatal sepsis due to *C. tropicalis*, and the death of one twin as an unfortunate outcome of twin pregnancy[81-83]. The imputable role of diabetes mellitus in the development of *Candida* chorioamnionitis after VVC (with potentially serious sequelae for the fetus) cannot be overstated.

Candidiasis in the urinary tract of diabetic patients

Urinary tract infections (UTI) are much more common in individuals with diabetes, and this is also valid for potential complications such as emphysematous cystitis, pyelonephritis, and kidney abscesses[84, 85]. Furthermore, type 2 diabetes mellitus is a well-recognized risk factor for both community- and

healthcare-associated acquired UTIs, but UTIs are linked to catheterization and following renal transplantation. In all of these scenarios, different *Candida* species have a prominent role[6,86]. In addition, in patients with diabetes, the duration of disease and poor glycoregulation in the long run lead to changes in the kidney's microvasculature and frequent polyuria/glycosuria, which can predispose them to more frequent urinary tract infections[87].

Delineating candiduria from frank UTI is still a controversial topic, as there are no steadfast laboratory criteria. Furthermore, *Candida* is a recognized commensal of the urogenital tract. Therefore, its presence in the urine sample adds ambiguity to making a definitive diagnosis of *Candida* UTI[88]. A further issue is that candiduria by itself may be the sole indicator of invasive candidiasis, with potentially serious outcomes (particularly in immunocompromised patients)[88]. In any case, the prevalence of candiduria in individuals with type 2 diabetes mellitus ranges between 2.27% and 30.00% in studies conducted worldwide, with notably higher rates in females[89] (Table 3).

A study from Ethiopia found significant candiduria in 7.5% of asymptomatic and 17.1% of symptomatic patients presenting with diabetes, with *C. albicans*, *C. glabrata,* and *C. tropicalis* being the most commonly implicated species[90]. In one study by Falahati *et al*[91] from Iran, uncontrolled diabetes, increased fasting blood sugar levels, and glucose in urine were all significantly related with candiduria, with the most frequent species being *C. glabrata* and *C. albicans* in 50.0% and 31.6% of cases, respectively, followed by *C. krusei*, *C. tropicalis*, and *C. kefyr*. This was corroborated by another study from Iran, where the candiduria rate was also high in individuals with type 2 diabetes mellitus that presented with inadequate blood glucose control[89]. The most frequently isolated species in the latter study was *C. albicans* (47.5%), followed by *C. glabrata* (37.5%), *C. kefyr* (10.0%), and *C. krusei* (5.0%)[89]. A recent study from Iran on 1450 urine samples highlighted diabetes as the most frequent risk factor for the development of candiduria and the three most common species as *C. albicans*, *C. glabrata,* and *C. tropicalis*[92].

Emphysematous cystitis is a rare complication that is almost exclusively seen in diabetic patients, while fungal microorganisms are seldom implicated in its pathogenesis[6]. Still, uncontrolled diabetes is viewed as a major risk factor for an increasing role of *Candid*a species (especially non-*albicans* species) in this specific pathology. Wang *et al*[93] described the case of a 53-year-old man with diabetes from China who presented with two rare and concomitant complications of *C. tropicalis* infection in the urinary tract: a discrete mass known as a fungus ball and emphysematous cystitis. Another study from the United States presented a case of a 49-year-old male with diabetes and emphysematous pyelitis caused by *C. tropicalis*, where early diagnosis and treatment led to a favorable outcome[94].

Treatment of candidiasis in patients with diabetes

Antifungal therapy is often not justified, even in UTIs caused by different types of *Candida*[95]. The assumption is that people with predisposing factors (*e.g.*, diabetes) should first be treated, which may in turn resolve the infection[96]. For individuals who have symptomatic UTIs caused by *Candida* spp. and when it is assumed that predisposing factors have been eliminated or at least kept to a minimum, the use of fluconazole is recommended due to the possibility of achieving high concentrations in urine. It can be administered orally, 200-400 mg daily, in a single dose for 14 d. Exceptions are infections caused by *C. krusei* and *C. glabrata*, where amphotericin B deoxycholate is often used (due to inadequate urine concentrations of other azole antifungals and echinocandins)[95]. In instances of resistant *Candida* spp. or in high-risk patients, amphotericin B is given intravenously at a dose of 0.3 to 0.6 mg/kg per day in the case of cystitis and given intravenously in a dose of 0.5 -0.7 mg/kg in the case of pyelonephritis[97]. In the case of resistant pyelonephritis, 25 mg/kg of flucytosine is added orally four times a day. The standard treatment regimen is 2 wk. The patient's kidney function should be taken into account[98]. The use of flucytosine, although very effective in the eradication of *Candida* spp., requires extra caution due to the toxicity it possesses[98]. If used alone, resistance to it occurs very quickly, and therefore therapy is not carried out longer than 7-10 d. Also, the drug is administered every 6 h at a dose of 25 mg/kg[99]. It is important to note that the recurrences of infections caused by *Candida* spp. are very common[100].

CONCLUSION

In conclusion, there is an increasing body of evidence that shows how patients with diabetes (particularly those characterized by unsatisfactorily controlled glycemia) are vulnerable to urogenital mycotic infections with *C. albicans* and other non-*albicans Candida* species of increasing importance. We have highlighted virulence factors of *C. albicans* and shown how the interplay of many pathophysiological factors can give rise to VVC with increased risk of recurrent episodes and dire pregnancy outcomes. There is also an increased risk of candiduria and UTI development caused by species of *Candida* in females and males alike (with the possibility of further complications such as emphysematous cystitis) as well as balanitis and balanoposthitis in (primarily uncircumcised) males. With a steadily increasing global burden of diabetes, these clinical conditions will undoubtedly become more prevalent in the future. All of this underscores the importance of establishing and preserving euglycemia, alongside any introduced antifungal treatment approaches, if our end-goal is to successfully manage urogenital

candidiasis in affected individuals with diabetes. Moreover, in order to minimize this high burden of yeast infections in individuals with diabetes, it is pivotal to identify those at high risk for developing type 2 diabetes mellitus and forestall the rise of complications; consequently, many lifestyle interventions (such as dietary changes, exercise, and weight reduction) have a much better impact than pharmacologic treatment. If the condition arises and the patient is faced with urogenital *Candida* infections, an early and appropriate treatment regimen should be introduced, especially to avoid several complicated conditions, which we have described.

FOOTNOTES

Author contributions: Talapko J substantially contributed to the conception and design of the article, interpretation of relevant literature, article drafting, and table preparation; Meštrović T substantially contributed to the conception and design of the article, interpretation of relevant literature, article drafting, and figure preparation; Škrlec I coordinated the literature search and article preparation and revised the manuscript critically for important intellectual content; All authors have read and approved the final version of the manuscript.

Conflict-of-interest statement: All authors report no relevant conflict of interest for this article.

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S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 822-834

DOI: 10.4239/wjd.v13.i10.822

ISSN 1948-9358 (online)

REVIEW

Everything real about unreal artificial intelligence in diabetic retinopathy and in ocular pathologies

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Specialty type: Ophthalmology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Hanada E, Japan; Jheng YC, Taiwan

Received: May 25, 2022
Peer-review started: May 25, 2022
First decision: August 1, 2022
Revised: August 11, 2022
Accepted: September 9, 2022
Article in press: September 9, 2022
Published online: October 15, 2022

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Abstract

Artificial Intelligence is a multidisciplinary field with the aim of building platforms that can make machines act, perceive, reason intelligently and whose goal is to automate activities that presently require human intelligence. From the cornea to the retina, artificial intelligence (AI) is expected to help ophthalmologists diagnose and treat ocular diseases. In ophthalmology, computerized analytics are being viewed as efficient and more objective ways to interpret the series of images and come to a conclusion. AI can be used to diagnose and grade diabetic retinopathy, glaucoma, age-related macular degeneration, cataracts, IOL power calculation, retinopathy of prematurity and keratoconus. This review article intends to discuss various aspects of artificial intelligence in ophthalmology.

Key Words: Artificial intelligence; Diabetic retinopathy; Deep learning; Machine learning; Ophthalmology

Core Tip: It is said that necessity is the mother of all inventions and converging global trends make multiplying eye care efficiency an increasingly urgent necessity. Artificial intelligence refers to an artificial creation of human-like intelligence of computer machines that can learn, reason, plan, perceive or process natural language.

Citation: Morya AK, Janti SS, Sisodiya P, Tejaswini A, Prasad R, Mali KR, Gurnani B. Everything real about unreal artificial intelligence in diabetic retinopathy and in ocular pathologies. *World J Diabetes* 2022; 13(10): 822-834
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/822.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.822

INTRODUCTION

Artificial intelligence (AI) refers to a machine's ability to mimic human cognitive functions, such as learning, reasoning, problem-solving, knowledge representation, social intelligence and general intelligence. It represents a significant advance in computer science and enables doing tasks using a computer with little human mind involvement following human training. AI was developed in the 1940s, but major advances ensued during the 1990s with significant improvements in machine learning, multi-agent planning, case-based reasoning, scheduling, data mining, natural language understanding and translation, vision, virtual reality, games, *etc*. Researchers have created an algorithm that can guess whether patients with cardiovascular diseases have lived or died based on their condition within a year. The algorithm could predict patient survival in 85% of cases based on data obtained by measuring the heart's electrical activity using electrocardiography. The rapid development in AI technology requires physicians and computer scientists to have a good mutual understanding of the technology and the medical practice to improve medical care. This review article presents the role of AI in various fields of ophthalmology.

METHODOLOGY

We searched highly cited articles in PubMed, Scopus database, Google Scholar, Web of Science, Cochrane library and Embase database on Artificial - Intelligence in Diabetic - Retinopathy, Age-related macular degeneration, Glaucoma, Keratoconus, Cataract, Dry Eye and other common ocular diseases published between the year 2000 to 2021. We also used Reference Citation Tool (RCA) for searching the keywords and articles were ranked based on the "Impact Index Per Article." The latest highlighted articles were selected for review. Only articles published in English were considered and the rest were rejected.

ARTIFICIAL INTELLIGENCE BASICS

Machine learning

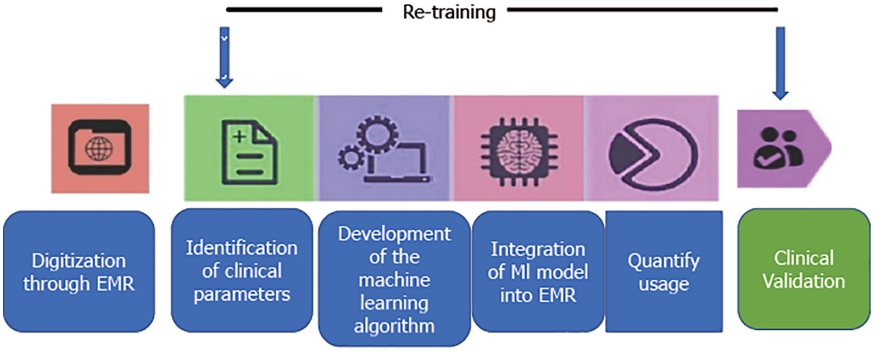
Machine learning (ML) is a core AI branch that aims to provide computers with the ability to learn without being explicitly programmed[1]. ML focuses on developing algorithms that can analyze data and make predictions[2] (Figure 1).

Deep learning

Deep learning (DL) differs from ML in that DL uses neural networks for making predictions and decisions. These neural networks were inspired by the biological neural networks of animal brains. They use the statistical probability principle derived from large data volumes to learn how to improve their accuracy, making DL a valuable tool for aiding physicians in clinical practice.

Generative adversarial network

Generative adversarial networks (GANs) are paired neural networks used for unsupervised ML. They can generate images or other data for the discriminative neural network to evaluate the data and provide feedback to aid the learning process[3].



DOI: 10.4239/wjd.v13.i10.822

Figure 1 Steps of machine learning algorithms that forms to analyze data and make predictions.

ARTIFICIAL INTELLIGENCE PLATFORMS

Algorithms resemble the AI software, whereas platforms resemble the computer hardware in which algorithms are installed and work to predict and make decisions. AI platforms simulate cognitive functions of the human mind including learning, reasoning, problem-solving, social intelligence and general intelligence[4].

Top Artificial intelligence platforms

The top AI platforms include Google, Microsoft Azure, TensorFlow, Railbird, Infosys Nia, Wipro HOLMES, Premonition, Dialogflow, Ayasdi, MindMeld, Meya, KAI and Vital A.I. Following the initial learning steps, the system or machine is taught to advance its initial learning for more accuracy and efficiency. This learning is further compounded by using complex mathematical equations to understand nonlinear relationships between different variables through an information flow called "neural networks." This "higher training" form enables AI to judge and weigh different outcome possibilities.

USE OF ARTIFICIAL INTELLIGENCE IN OPHTHALMOLOGY

From the back of the eye to the front, AI is expected to provide ophthalmologists with novel automated tools to diagnose and treat ocular diseases. Recently, the application of AI in medicine has garnered much attention from big players in the digital world, such as Google and IBM. This is expected to stimulate research and development for disease diagnosis and treatment. Researchers in the field of AI ophthalmology view computerized analytics as the path toward more efficient and objective ways of image interpretation compared with modern eye care practice.

Diabetic retinopathy

Patients with diabetes require regular and repetitive screening to detect and treat diabetic retinopathy (DR)[4,5]. Conventionally, this screening is performed by dilated fundus examination or color fundus photography using conventional fundus cameras (mydriatic or nonmydriatic). The primary issue in this screening is retinal image grading by retinal specialists or trained personnel, who are few compared with the patient load requiring screening. Another problem is that most patients reside in rural areas. Finally, constant follow-ups are needed for several years[4].

DR, a complication of chronic diabetes, is a vasculopathy affecting one-third of patients with diabetes and possibly leading to irreversible blindness[6,7]. Most AIs have been evaluated for their application in DR detection with the primary goal of assisting the development of a mass and rapid screening tool with high sensitivity and specificity. Considering the huge diversity in the clinical presentation of DR, it is essential for an AI neural network to be multilayered and extensively trained. This requires the use of multiple images evaluated against the ground truth.

Most studies have used the International Clinical Diabetic Retinopathy Disease severity scale, a 5-point scale [no apparent retinopathy, mild non-proliferative DR (NPDR), moderate NPDR, severe NPDR and proliferative diabetic retinopathy (PDR)]. Referable DR is defined as moderate or severe DR as disease management often changes from yearly screening to closer follow-up for moderate disease severity. A recent study by Shah *et al*[8] used an AI algorithm with a deep convolutional neural network (DCNN). It assessed its sensitivity and specificity with double validation, *i.e.* external and internal validation. External validation was performed using the Methods to Evaluate Segmentation and Indexing Techniques in the Retinal Ophthalmology dataset, *i.e.* the MESSIDOR dataset. In contrast,

internal validation was performed by two retinal specialists. The main advantage of this study was that 112489 images, acquired from various fundus cameras taking pictures of both mydriatic and nonmydriatic eyes, were fed into AI, thereby giving a multiethnicity advantage to the dataset. The agreement between AI and internal/external validation was high for ANY DR and REFERRAL DR, with a sensitivity of > 95%. The agreement for sight-threatening DR between AI and external validation was high but moderate between AI and internal validation. However, this did not affect the conclusion that AI proved to be a useful screening tool and detected referral DR cases with high specificity.

Valverde *et al*[9] reviewed the available algorithms and detailed the methods for segmenting exudates, red lesions and screening systems. These segmentation methods were used to develop a computer-aided diagnosis for automated DR detection, such as Retmarker DR, Retinalyze System, IDx-DR (first FDA-approved system), iGradingM and Telemedical Retinal Image Analysis and Diagnosis Network. Overall, all these systems achieved high sensitivity and specificity, provided that the segmentation of exudates was used to screen for DR rather than the segmentation of red lesions. Medios, an offline AI, was developed and studied by Sosale *et al*[10]. This offline algorithm was created because of Internet access limitations and the high computational power required for all cloud-based AIs in a developing country. Fundus photographs were captured using Remidio Non-Mydriatic Fundus on Phone 10 (NM FOP 10) and image processing was directly performed on the smartphone graphics processing unit. The sensitivity and specificity of the AI algorithm for detecting referral DR were 98% and 86%, respectively. For any DR, its sensitivity and specificity were 86% and 95%, respectively. Compared with other online cloud-based software, such as EyeArt and IDx-DR, Medios had better sensitivity and equivalent specificity (Figure 2). The specific abnormalities that can be detected using continuous machine learning (CML) are macular edema[11,12], exudates[13], cotton-wool spots[14], microaneurysms and optic disc neovascularization[15]. Commercially available DR detection and analysis technologies include Retinalyze System, IDx-DR, iGradingM and RetmarkerDR. The difference is that only a few modalities use lesion-based grading, whereas the others use image-based grading. The sensitivity of this system has reached around 80%, but its specificity remains lower than 90%.

A DL GAN can be trained to map anatomical features from different image modalities, *i.e.* fundus photographs and fluorescein angiography (FA) images, onto a shared feature manifold to generate one image modality from another[16]. Using GAN, detailed retinal vascular structures can be produced without the requirement of FA to avoid its potential side effects. The inferred structural measurements of retinal vasculature may allow clinicians to identify the natural history of changes in the retinal vasculature and the clinical outcomes of retinal diseases, as previously reported by direct fundus image analysis, but with the accuracy of FA or even optical coherence tomography angiography image analysis[17].

Morya *et al*[18] evaluated the first smartphone-based online annotation in the world, a tool for rapid and accurate image labeling, using AI-based DL for DR. This DL model evaluated its accuracy based on a binary referral DR classification system, depending on whether a retinal image had referral DR or not. A total of 32 ophthalmologists used the tool for over 55000 images. The data analysis proved considerable flexibility and portability with favorable grader variability in concurrence with image annotation. Table 1 demonstrates the collective data of various studies on DR-related AI. This table has been reproduced from the article by Padhy *et al*[19].

AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is the cause of approximately 9% of cases of blindness globally[20]. The worldwide number of people with AMD was projected to be 196 million in 2020, which is expected to substantially increase to 288 million in 2040[20]. The age-related eye disease study (AREDS) developed a simplified severity scale for AMD[22]. This scale combines the risk factors from both eyes to generate an overall score for the individual based on the presence of one or more large drusen (diameter of > 125 mm) or AMD pigmentary abnormalities in the macula of each eye. The simplified severity scale is also clinically useful because it allows ophthalmologists to predict an individual's 5-year risk of developing late AMD[23]. AMD detection and prediction are essential for individualized treatment. Using AI in cases of AMD could increase the detection rate of lesions such as drusen, with the presence of fluid and reticular pseudo-drusen and geographic atrophy.

Several DL systems have been developed for classifying color fundus photographs based on AMD severity scales. These severity scales include referable and non-referable AMD[22] and multiclass AMD classification systems (*e.g.*, 9-step AREDS severity scale and 4-class). Recent studies have shown the robust performance of automated AMD classification systems based on optical coherence tomography (OCT) scans[24].

DeepSeeNet is based on color fundus photography and uses three networks-Drusen-Net, Pigment-Net and Late AMD-Net (Figure 3). These three networks were designed as DCNNs, each with an Inception-v3 architecture and a state-of-the-art convolutional neural network (CNN) model for image classification. Similar to the study by De Fauw *et al*[25], DeepSeeNet includes two stages by design for improved performance and increased transparency. Images were obtained from the AREDS dataset,

Table 1 Comparative analysis of various studies done on artificial intelligence in diabetic retinopathy[19]

Ref.	Sensitivity, specificity or accuracy of the study	Total fundus images examined	Types of AI used	Main objective
Wong *et al*[20]	Area under the curve were 0.97 and 0.92 for microaneurysm and hemorrhages respectively	143 images	A three-layer feed forward neural network	Deals with detecting the microaneurysm and hemorrhages. Frangi filter used
Imani *et al*[57]	Sensitivity of 75.02%-75.24%; Specificity of 97.45%-97.53%	60 images	MCA	Detected the exudation and blood vessel
Yazid *et al*[58]	97.8% in sensitivity, 99% in specificity and 83.3% in predictivity for STARE database. 90.7% in sensitivity, 99.4% in specificity and 74% in predictivity for the custom database	30 images	Inverse surface thresholding	Detected both hard and soft exudates
Akyol *et al*[59]	Percentage accuracy of disc detection ranged from 90%-94.38% using different data set	239 images	Key point detection, texture analysis, and visual dictionary techniques	Detected the optic disc of fundus images
Niemeijer *et al*[13]	Accuracy in 99.9% cases in finding the disc	1000 images	Combined k-nearest neighbor and cues	Fast detection of the optic disc
Rajalakshmi *et al*[60], Smart phone based study	95.8% sensitivity and 80.2% specificity for detecting any DR. 99.1% sensitivity and 80.4% specificity in detecting STDR	Retinal images of 296 patients	Eye Art AI Dr screening software used	Retinal photography with Remidio 'Fundus on Phone'
Eye Nuk study	Sensitivity was 91.7%; Specificity was 91.5%	40542 images	Eye PAC Stelescreening system	Retinal images taken with traditional desktop fundus cameras
Ting *et al*[61]	Sensitivity and specificity for RDR was 90.5% and 91.6%; For STDR the sensitivity was 100% and the specificity was 91.1%	494661 retinal images	Deep learning system	Multiple Retinal images taken with conventional fundus cameras
IRIS	Sensitivity of the IRIS algorithm in detecting STDR was 66.4% with false-negative rate of 2% and the specificity was 72.8%. Positive Predictive value of 10.8% and negative predictive value 97.8%	15015 patients	Intelligent Retinal Imaging System (IRIS)	Retinal screening examination and nonmydriatic fundus photography

This table has been reproduced from the article by Padhy *et al*[19]. Citation: Padhy SK, Takkar B, Chawla R, Kumar A. Artificial intelligence in diabetic retinopathy: A natural step to the future. *Indian J Ophthalmol* 2019; 67: 1004-1009. Copyright© The Authors 2019. Published by *Indian Journal of Ophthalmology*. The authors have obtained the permission for table using from the Indian Journal of Ophthalmology Publishing Group (Supplementary material). AI: Artificial intelligence; MCA: Morphological component analysis.

comprising approximately 60000 retinal images. DeepSeeNet operates by first detecting individual risk factors (drusen and pigmentary abnormalities) in each eye and then combining values from both eyes to assign an AMD score for the patient. Therefore, DeepSeeNet closely matches the clinical decision-making process (Figure 3). The accuracy of Fine-Tuned DeepSeeNet (FT-DSN) was superior to that of human retinal specialists (67% *vs* 60%). On further analysis, the overall accuracy of FT-DSN was superior. However, subgroup analysis showed that FT-DSN correctly classified participants with severity scale scores of 0-4 more often than the retinal specialists. In contrast, the retinal specialists correctly classified those with late AMD more often than FT-DSN. Lee *et al*[26] developed an AMD screening system to differentiate between normal and AMD OCT images. They trained their CML using 48312 normal and 52690 AMD images. Their CML had a peak sensitivity and specificity of 92% and 93%, respectively. Treder *et al*[27] used OCT images (1112 images) to develop a CML that could differentiate a healthy macula from a macula showing exudative AMD, with a sensitivity of 100% and a specificity of 92%.

Bogunovic *et al*[28] developed a data-driven interpretable predictive model to predict the progression risk in those with intermediate AMD. Drusen regression, an anatomic intermediate AMD endpoint, and advanced AMD onset can be predicted using this specifically designed, fully automated, ML-based classifier. Treder *et al*[27] fed corresponding OCT images of patients with low or high anti-vascular endothelial growth factor (VEGF) injection requirements into a random forest (RF) classifier to develop a predictive model. The treatment requirement prediction showed an area under the curve (AUC) of 70%-80%. Prahs *et al*[29] trained a DCNN on OCT images to facilitate decision-making regarding anti-VEGF injection, and the outcomes were better than those using CML. These studies are an essential step toward image-guided prediction of treatment intervals in neovascular AMD or PDR management. In addition to screening, some studies have focused on grading AMD and predicting visual acuity from OCT images. This will aid clinicians in formulating a visual prognosis and support them in their

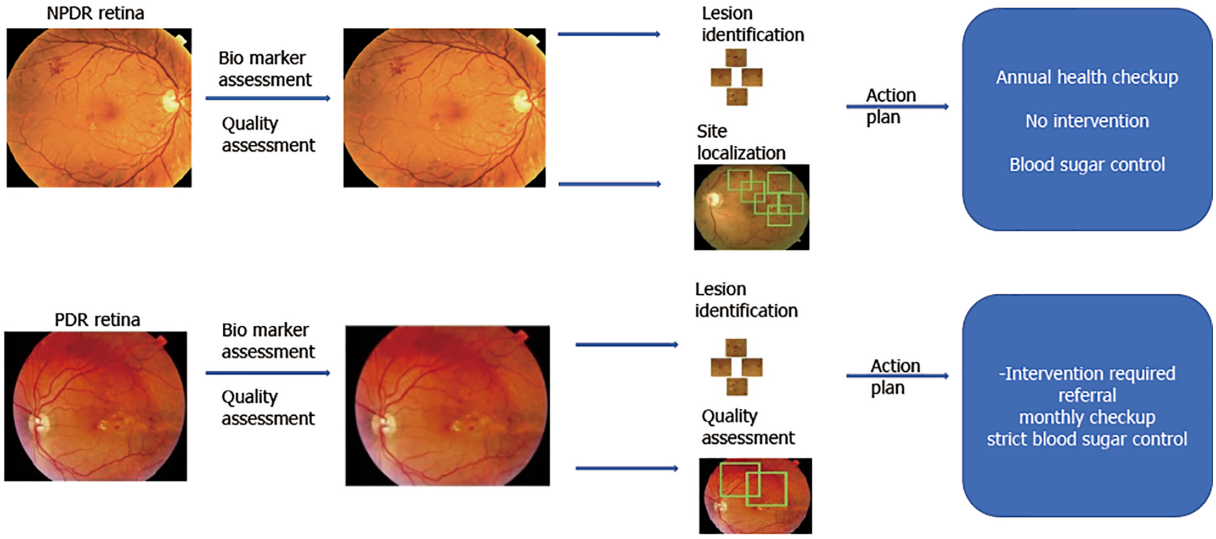


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Figure 2 AI software assess the diabetic retinopathy into referrable and non-referrable interventions.

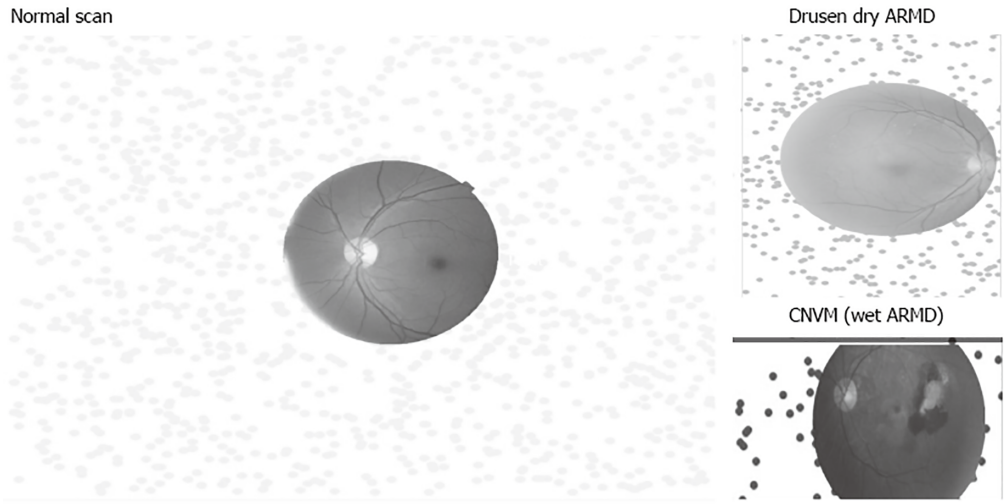

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Figure 3 Deep Sea net classify age-related macular degeneration (AMD) into dry AMD and wet AMD based on fundus photograph.

decision-making. Aslam *et al*[30] and Schmidt-Erfurth *et al*[31] developed CMLs that could estimate visual acuity. Aslam *et al*[30] trained their CML on data from 847 OCT scans, whereas Schmidt-Erfurth *et al*[31] trained their CML on data from 2456 OCT scans (from 614 eyes).

AI systems can be trained to perform segmentation, classification and prediction using retinal OCT images. Several AI systems were demonstrated to display high accuracy for segmentation which is essential to quantify intraretinal fluid, subretinal fluid and pigment epithelial detachment. Compared with noncomputerized segmentation techniques, the DL algorithm developed by Lee *et al*[26] accurately differentiated fluid accumulation from other abnormal retinal findings. Further, De Fauw *et al*[25] confirmed the ability of DL to detect > 50 retinal conditions and the robustness of the AI system in triaging the urgency of referrals for patients with retinal diseases. Table 2 is a summary of AI algorithms used for AMD.

Glaucoma

Glaucoma, also known as the silent sight killer, is the leading cause of preventable and irreversible blindness worldwide. The disease remains asymptomatic and an estimated 50%-90% of individuals with glaucoma remain undiagnosed. Thus, glaucoma screening is recommended for its early detection and treatment. Cup-disk ratio (CDR) can be calculated to assist early-stage glaucoma diagnosis using AI models[32]. After locating the coarse disk margin using a spatial correlation smoothness constraint, a support vector machine (SVM) model is trained to find the patches on OCT images to identify a

Table 2 Summary of artificial intelligence algorithm used in age-related macular degeneration

Ref.	Sensitivity	Specificity	Diagnostic accuracy	Output
Grassman *et al*[62]	84.20	94.30	63.3, Kappa of 92%	Final probability value for referable *vs* not referable
Ting *et al*[61]	93.20	88.70	Area under curve-0.932	Identifying referable AMD and advanced AMD
Lee *et al*[26]	84.60	91.50	87.60	Prediction of binary segmentation map
Treder *et al*[27]	100	92	96	AMD testing score-score of 0.98 or greater adequate for diagnosis of AMD

AMD: Age-related macular degeneration.

reference plane that can calculate the CDR[33].

In 2013, Yousefi *et al*[16] published an AI study on the progression of primary open-angle glaucoma (POAG) in 180 patients using many different CMLs and independent features. They found that retinal nerve fiber layer features provided sufficient information for CMLs to differentiate between stable POAG and progressing POAG at an early-moderate disease stage. RF tree and lazy K star were the most sensitive CMLs. Chen *et al*[34] developed a CNN using two different datasets [ORIGA dataset: 650 images (99 for training and 551 for validation) and SCES dataset: 1676 images (entirely used for validation as the images in the ORIGA set were used for training)] to detect POAG based on optic disk images. They reported the area under the receiver operating characteristic curve values of 0.831 and 0.887 for ORIGA and SCES datasets, respectively. Kim *et al*[35] and Raghavendra focused on detecting glaucoma *vs* normal fundus images. They reported an accuracy of 87.9%, equivalent to the accuracy of human experts, demonstrating an efficient method for glaucoma screening. Raghavendra *et al*[36] tested their CML on 589 normal and 837 glaucoma images and obtained a score of 0.98 for sensitivity, specificity and accuracy.

DL performs better than CML in detecting pre-perimetric open-angle glaucoma[36]. Holistic and local features of the optic disc on fundus images have been used to mitigate the influence of optic disk misalignment for glaucoma diagnosis[37]. Li *et al*[38] demonstrated that DL could be used to identify referable glaucomatous optic neuropathy with high sensitivity and specificity. Table 3 is a summary of studies using AI to detect progression in eyes with glaucoma.

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a leading cause of treatable childhood blindness, provided it is diagnosed timely[39]. This disease necessitates strict follow-up and screening which are very tedious and demanding. Repeated ROP screening and follow-up consume substantial manpower and energy. Therefore, the application of AI in ROP screening may improve the efficiency of care for ROP.

Wang *et al*[40] developed an automated ROP detection system called DeepROP using deep neural networks (DNNs). ROP detection was divided into ROP identification and grading tasks. Two specific DNN models–Id-Net and Gr-Net–were designed for the identification and grading tasks, respectively. Id-Net achieved a sensitivity of 96.62% and a specificity of 99.32% for ROP identification, whereas Gr-Net attained a sensitivity of 88.46% and a specificity of 92.31% for ROP grading. In another 552 cases, the developed DNNs outperformed some human experts[41].

A similar AI, developed by Tan Z, achieved similar accuracy for detecting plus ROP. They reported that this AI could distinguish the plus disease with 95% accuracy, comparable to the diagnoses of experts and much more precise than those of non-experts. Various studies have reported promising results, most of which were based on two-level sorting (plus or not plus disease).

Keratoconus

There are significant obstacles in distinguishing patients with very early keratoconus signs from the normal population. This is attributed to the limited availability of samples owing to low disease prevalence. For this purpose, the application of AI in corneal topography interpretation has been attempted. The methods used discriminative classifiers that, given a set of independent machine-derived variables from corneal topography (*e.g.*, simulated K readings and topographic asymmetries), can be trained to differentiate between two or more classes of topography (*e.g.*, normal, astigmatic and keratoconus).

AI has been used to detect keratoconus and forme fruste keratoconus[42] based on data from Placido topography, Scheimpflug tomography[43], anterior segment spectral domain OCT and biomechanical metrics (CorvisST and corneal hysteresis). Further, data from Pentacam[44], Sirius[45], Orbscan II, Galilei and TMS-1 have been studied using ML algorithms to detect early keratoconus.

The Pentacam RF index (PRFI) is an RF model built using data from Pentacam HR (Oculus, Wetzlar, Germany). It was the only model trained using the preoperative examination data of patients that developed ectasia. The index already available on the device (BAD-D) presented a sensitivity of 55.3%, whereas PRFI identified 80% of the cases correctly. In the external validation set, the model showed an

Table 3 Summary of studies using artificial intelligence to detect progression in Glaucomatous eyes

Ref.	No. of eyes	Instrument	Approach	Comments
Lin *et al*[63]	80	SAP	Supervised ML	Sensitivity-86%; Specificity-88%
Goldbaum *et al*[64]	478 suspects; 150 glaucoma; 55 stable glaucoma	SAP	Unsupervised ML	Specificity-98.4%, AROC not available; Use of variational Byesian. Independent component analysis mixture model in indentifying patterns of glaucomatous visual field defects and its validation
Wang *et al* [65]	11817 (method developing cohort) and 397 (clinical evaluation cohort)	SAP	Unsupervised ML	AROC of the archetype method 0.77
Yousefi *et al* [16]	939 Abnormal SAP and 1146 normal SAP in the cross section and 270 glaucoma in the longitudinal database	SAP	Unsupervised ML	Sensitivity 34.5%-63.4% at specificity 87% Comment: it took 3.5 years for ML analysis to detect progression while it took over 3.5 years for other methods to detect progression in 25% of eyes
Belghith *et al*	27- progressing; 26-stable glaucoma and 40 healthy controls		SD OCT Supervised ML	Sensitivity -78% Specificity in normal eyes-93%; 94% in non-progressive eyes

ML: Machine learning; OCT: Optical coherence tomography.

accuracy of 85% for detecting the normal topographic eye of very asymmetric cases (VAE-NT), reaching a specificity of 96.6%[46].

A single decision tree method was proposed based on the data obtained from the Galilei Dual Scheimpflug Analyzer (Ziemer Ophthalmic Systems AG, Port, Switzerland). This index showed a sensitivity of 90% and a specificity of 86% for detecting early disease forms[47]. Discriminant linear models were also successfully used to analyze the data obtained from Orbscan II (Technolas, Munich, Germany) with a sensitivity of 92% and a specificity of 96% in the first validation set and a sensitivity of 70.8%, and a specificity of 98.1% in a different ethnic background population[48].

Ambrósio *et al*[48] evaluated AI-based tomographic and biomechanical index (TBI), which combines Scheimpflug-based corneal tomography and biomechanics (Corvis ST) for improving ectasia detection. The Kerato Detect algorithm analyzes the corneal eye topography using a CNN that can extract and learn the features of a keratoconus eye. The results ensure high-level performance yielding an accuracy of 99.33% for the test dataset. Neural networks have been used to evaluate the waveform signals of the Ocular Response Analyzer (Reichert Ophthalmic Instruments, Buffalo, United States) yielding high accuracy for the study validation sample comprising early keratoconus forms (AUC, 0.978). The RF model called TBI achieved a sensitivity of 90.3% and a specificity of 96% for detecting VAE-NT. The combination of tomographic and biomechanical parameters was superior to either method used alone.

Sharif *et al*[49] showed that corneal images obtained *via* confocal microscopy could be assessed in detail using a committee machine developed from artificial neural networks and adaptive neuro-fuzzy inference systems that can detect abnormalities with high accuracy and enable 3D visualization. Nevertheless, considering that the research on these aspects is limited, there is a possibility that the characteristics learned in AI training may not be similar to those in another clinical population. When using tomographic data rather than Placido topographic data, researchers have found that combining biomechanical or additional imaging data is necessary to enhance the performance for detecting early keratoconus signs.

CORNEAL DYSTROPHIES AND DYSPLASIA

Eleiwa *et al*[50] used AI to differentiate Fuchs endothelial corneal dystrophy (FECD, without corneal edema) from late-stage FECD (with corneal edema) based on high-definition OCT images. The model they developed had a sensitivity of 99% and a specificity of 98% in differentiating normal cornea from FECD (early or late).

Gu *et al*[51] reported an AUC of 0.939 for detecting corneal dystrophy or degeneration using a slit-lamp photograph-based DL model. They included ocular surface disorders such as limbal dermoid, papilloma, pterygium, conjunctival dermolipoma, conjunctival nevus and conjunctival melanocytic tumors to differentiate ocular surface neoplasms. However, considering the limited existing evidence, the use of AI for detecting ocular surface neoplasms warrants further exploration. Kessel *et al*[52] created trained DL algorithms to detect and analyze amyloid deposition in corneal sections in patients with familial amyloidosis undergoing full-thickness keratoplasty.

Dry eye

Dry eye disease is a common condition that affects 8% of the global population and is caused by the reduced quantity or quality of tears. Left untreated, dry eye can result in pain, ulcers and even corneal

scars. Therefore, rapid diagnosis is essential and clinically based on tear production measurement and a tear film stability evaluation.

In a recent study, researchers used infrared thermal images of the eye along with the ML algorithms Gabor transform and Discrete Wavelet Transform (DWT) to detect dry eyes[53]. These ML methodologies were used to extract features from specific image frames, further segmented into eye regions, and the data were analyzed accordingly. Principal component analysis was ranked using a t-value and fed into the SVM classifier. Using the 1st, 5th, and 10th after the first blink, they achieved classification accuracies of: (1) 82.3%, 89.2% and 88.2% for the left eye; and (2) 93.4%, 81.5% and 84.4% for the right eye, respectively. Similarly, using the 1st, 5th, and 10th frames of the lower half of the ocular region, they achieved accuracies of: (1) 95.0%, 95.0% and 89.2%; and (2) 91.2%, 97.0% and 92.2% for the left and right eyes, respectively. This study showed that the lower half of the ocular region is superior to the upper half of the ocular region.

This method offers several advantages, such as being semiautomatic and making it less susceptible to interobserver variability. It is more accurate than standard clinical tools, more convenient for the patient and does not require a special dye. Gabor transform and DWT are methodologies for automatic feature extraction from biomedical images.

Cataract

Cataract refers to the clouding of the eye lens. It is the leading cause of blindness worldwide. Therefore, automatic detection for the diagnosis of this disease will be cost-effective.

Srivastava *et al*[54] proposed a system that automatically grades the severity of nuclear cataracts based on slit-lamp images. First, the lens region of interest is identified, following which the CNN filters randomly selected image patches, generating local representations *via* an iteration process with random weights. They named it ACASIA-NC_v0.10 (*i.e.*, Automatic Cataract Screening from Image Analysis-Nuclear Cataract, version 0.10) and specifically used the "visibility cue" for nuclear cataract grading C. Their system used visible features of the nucleus, such as sutures and demarcation lines, in greyscale. With the help of the software, they could analyze the number of visible features. ACASIA-NC_v0.10 achieved a similarity of > 70% against clinical grading and reduced the error by > 8.5%. Other studies similar to Liu *et al*[55] mainly focused on identifying pediatric cataracts. They reported exceptional accuracy and sensitivity for lens classification and density. In addition, cataract grading can also be achieved automatically based on lens OCT findings.

SMARTPHONE-BASED APPS USING AI IN OPHTHALMOLOGY

The advantages of using smartphones are many, including having built-in internal data storage and cloud storage capabilities[56]. Pegasus VISULYTIX, an inexpensive smartphone clip-on optic nerve scanner expected to aid the diagnosis and treatment of those with chronic blinding diseases, such as glaucoma, using AI, is being adapted. Pegasus could detect glaucomatous optic neuropathy with an accuracy of 83.4%, comparable to the average accuracies of ophthalmologists (80.5%) and optometrists (80%) using the same images.

CC-Cruiser was developed to study the application of AI in congenital cataracts (CC). CCs cause irreversible vision loss and breakthroughs in the research on CCs have substantially contributed to the field of medicine. Researchers have developed a three-fold AI system that includes identification networks for CC screening in populations, evaluation networks for risk stratification of patients with CC and strategist networks to assist ophthalmologists in making treatment decisions.

Shaw created a ComputeR Assisted Detector LEukocoia (CRADLE) app that uses AI to identify white eyes indicative of several serious eye diseases. The sensitivity of CRADLE for detecting white eyes in children aged ≤ 2 years surpassed 80%, which was substantially higher than the sensitivity of physical examination (8%). This new smartphone app takes advantage of parents' fondness for snapping pictures of their children to identify signs of a severe eye disease that the child might be developing. On average, the app detected white eyes in pictures collected 1.3 years before diagnosis.

FUTURE OF ARTIFICIAL INTELLIGENCE APPLICATION

The AI-based platform provides an intelligent diagnosis of eye diseases at present. It focuses on binary classification problems, whereas visiting patients suffer multi-categorical retinal disorders in clinical settings. Multimodal clinical images, such as OCTA, visual field and fundus images should be integrated to build a generalized AI system for more reliable AI diagnosis. The challenge is coordinating multicenter collaborations to build good quality and extensive data collection to train and improve AI models. AI is an instrument to upturn clinical decision power with many possible applications for ophthalmologists.

LIMITATIONS OF ARTIFICIAL INTELLIGENCE

Any software design is not perfect, and so artificial intelligence is also not bias-proof. Five distinct types of machine learning bias that we need to be aware of and guard against: (1) Sample bias: poor data collection for training. Example: Labeling other vascular retinopathies as DR; (2) Prejudice bias: Prejudice bias results from training data that is influenced by stereotypes. For example, a large cup is always glaucoma; (3) Measurement bias. For example, fundus photo color, different cameras give different color measurements; best avoided by having multiple or similar measuring devices and humans trained to compare the output of these devices when developing the algorithm; (4) Algorithm bias: Choosing the wrong software algorithm for a specific disease; and (5) The quality control of images for prediction.

CONCLUSION

With the substantial advances in AI in the field of ophthalmology, it can be assumed that now is the dawn of AI in ophthalmology. With the advent of technologies based on different AI modules, such as DL, ML and GAN, it can be assumed that AI has a promising role in the diagnosis of DR, ARMD, dry eye, glaucoma, keratoconus and cataracts. In particular, these AI-based applications are more relevant during the present coronavirus disease 2019 era and for serving the remotest of areas worldwide. Compared with conventional tests performed at tertiary ophthalmic centers, AI performs better in the screening and diagnosis of various eye diseases. After considering all the facts and overcoming challenges in its application, it can be said that AI in the field of ophthalmology is here to stay and revolutionize eye care in the 21st century. Nonetheless, researchers in the field of ophthalmology need to develop more robust AI modules with better verification and validation. Further, we must not rely only on near-real AI as no modality can possibly replace the level of affection, care and sensitivity as that provided by human caregivers.

FOOTNOTES

Author contributions: Morya AK and Janti SS contributed equally to this work; Sisodiya P, Tejaswini A, Gurnani B, Prasad R and Mali KR designed the research study; Morya AK and Janti SS performed the research; Sisodiya P, Tejaswini A, Gurnani B contributed new search and analytic tools; Morya AK, Janti SS,Tejaswini A and Gurnani B analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

Conflict-of-interest statement: All authors report no relevant conflict of interest for this article.

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S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 835-850

DOI: 10.4239/wjd.v13.i10.835

ISSN 1948-9358 (online)

MINIREVIEWS

New therapeutic approaches for type 1 diabetes: Disease-modifying therapies

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Feng JF, China; He Z, China; Long P, China; Nong X, China

Received: May 28, 2022
Peer-review started: May 28, 2022
First decision: August 1, 2022
Revised: August 8, 2022
Accepted: September 15, 2022
Article in press: September 15, 2022
Published online: October 15, 2022

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Abstract

It has been 100 years since the first successful clinical use of insulin, yet it remains the only treatment option for type 1 diabetes mellitus (T1DM) patients. Advances in diabetes care, such as insulin analogue therapies and new devices, including continuous glucose monitoring with continuous subcutaneous insulin infusion have improved the quality of life of patients but have no impact on the pathogenesis of the disease. They do not eliminate long-term complications and require several lifestyle sacrifices. A more ideal future therapy for T1DM, instead of supplementing the insufficient hormone production (a consequence of β-cell destruction), would also aim to stop or slow down the destructive autoimmune process. The discovery of the autoimmune nature of type 1 diabetes mellitus has presented several targets by which disease progression may be altered. The goal of disease-modifying therapies is to target autoimmune mechanisms and prevent β-cell destruction. T1DM patients with better β-cell function have better glycemic control, reduced incidence of long-term complications and hypoglycemic episodes. Unfortunately, at the time symptomatic T1DM is diagnosed, most of the insulin secreting β cells are usually lost. Therefore, to maximize the salvageable β-cell mass by disease-modifying therapies, detecting autoimmune markers in an early, optimally presymptomatic phase of T1DM is of great importance. Disease-modifying therapies, such as immuno- and regenerative therapies are expected to take a relevant place in diabetology. The aim of this article was to provide a brief insight into the pathogenesis and course of T1DM and present the current state of disease-modifying therapeutic interventions that may impact future diabetes treatment.

Key Words: Type 1 diabetes; Mesenchymal stem cell; Immunotherapy; Islet cells; Autoimmunity; Regenerative medicine

Core Tip: Our knowledge is rapidly growing about the pathomechanism of type 1 diabetes mellitus, and new and improved therapies have emerged. However, the long-term complications and the required lifestyle changes cannot be eliminated. There is a growing number of research that aims to find specific immunological markers/targets that have a role in disease development. The ultimate goal is finding new therapeutic ways to treat the disease and to delay or even prevent its development. The aim of this review was to provide a brief insight into the current state of disease-modifying therapeutic interventions that may impact future diabetes care.

Citation: Nagy G, Szekely TE, Somogyi A, Herold M, Herold Z. New therapeutic approaches for type 1 diabetes: Disease-modifying therapies. *World J Diabetes* 2022; 13(10): 835-850
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/835.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.835

INTRODUCTION

For many years it was accepted that type 1 diabetes mellitus (T1DM) starts with the classical triad of polyuria, polydipsia and polyphagia. However, it became clear that T1DM is a long standing, progressive disease with a preclinical phase without symptoms and with the appearance of multiple T1DM-associated autoantibodies. The preclinical phase is followed by a symptomatic clinical phase[1]. The burden of living with the chronic disease is considerable for the patient, the family and the society. This minireview focused on the clinical applications of novel disease-modifying therapeutic intervention options in early stages of T1DM that may prevent or reverse clinically overt symptomatic T1DM. The presentation of the latest improvements available for middle and late stage disease and diabetic complications is out of the scope of the current review.

Immunopathogenesis of T1DM

The pathogenesis of T1DM involves a complex interaction between pancreatic β cells and the innate and natural immune systems. The precise mechanism that leads to the loss of immune tolerance is still unclear. However, viral infections, nutritional factors and the perinatal environment have been associated with the disease[2-4]. It is assumed that the stability of the trimolecular complex (T cell receptor/human leukocyte antigen/peptide) during thymic selection plays a major role in the escape of autoimmune T cells[5].

In the development of T1DM, the initial step of the destructive process is considered to be the uptake and presentation of β-cell-derived peptides, autoantigens, by the antigen-presenting cells. Next, antigen-presenting cells, which can be both macrophages and dendritic cells, migrate to lymph nodes around the pancreas and activate $CD4^+$ helper T cells (Th)[6]. Th cells differentiate into Th1, which have a proinflammatory phenotype. Th1 cells are the key effector cells in the pathogenesis of T1DM and are capable of producing interferon-γ, tumor necrosis factor α, interleukin 1 (IL-1) and IL-2. These cytokines inhibit Th2 polarization, the cells responsible for the protection of islets[7]. Th1 cells are necessary for the activation and recruitment of other autoreactive cells, such as $CD8^+$ cytotoxic T lymphocytes (CTL), which are responsible for the lysis/apoptosis of β cells presenting the major histocompatibility complex I autoantigen complex. The cell-destructive effect of activated CTLs is due to macromolecules stored in granules (*e.g.*, perforin, granzyme), to the cytokines and to caspase-dependent apoptosis[8].

B cells are stimulated by Th1 cells and produce autoantibodies against β cells [islet cell antibody, glutamic-acid-decarboxylase antibody (GADA), islet tyrosine phosphatase 2 antibody, insulin autoantibody and zinc transporter 8 antibodies]. These antibodies have become the biomarkers of T1DM [9]. Furthermore, Th1 cells enhance antigen presenting, costimulatory and effector functions of macrophages and dendritic cells. Natural killer cells also contribute to β-cell destruction through their cytolytic effects and antibody-dependent cellular cytotoxicity. Th17, with strong inflammatory effects, is also involved in the inflammatory process: It secretes IL-17 family cytokines and plays an important role in neutrophil granulocyte recruitment and activation[10].

Under ideal conditions, regulatory T cells (Treg) inhibit the autoreactive lymphocytes. If Treg cells are deficient, the rate of T1DM progression is increased[11]. The above-mentioned immune cells infiltrate the islets (insulitis) and eventually cause β-cell death and reduced insulin levels (Figure 1).

New staging classification system of T1DM

T1DM is the result of a destructive autoimmune-mediated process in which insulin-producing β cells in the islets of Langerhans are damaged. According to the novel staging classification system proposed by

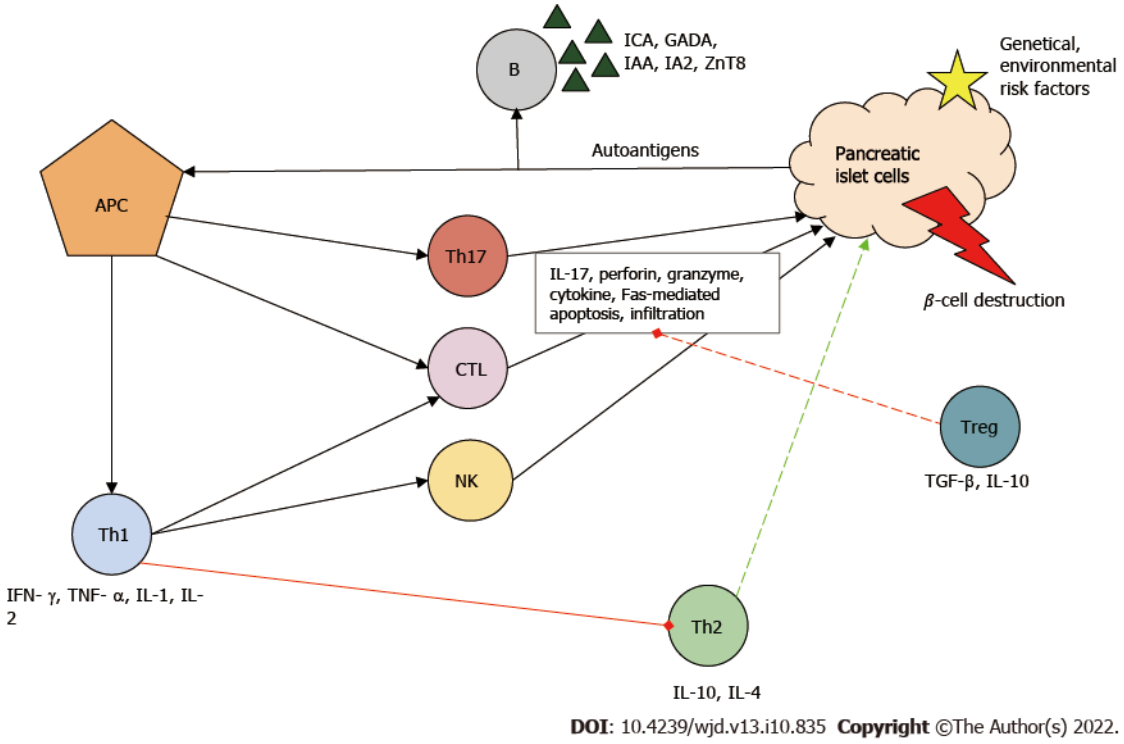
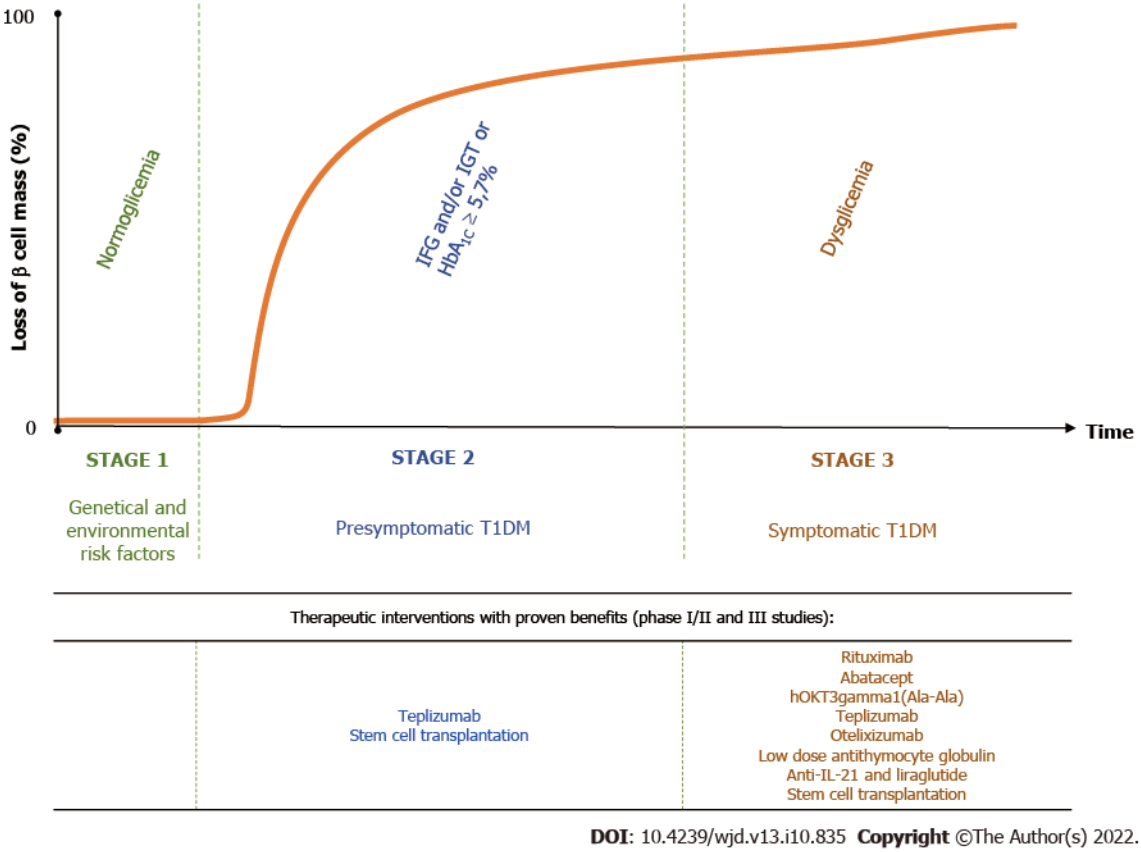


Figure 1 Immunopathogenesis of type 1 diabetes mellitus. Autoantigens from pancreatic islet β-cells are presented by antigen-presenting cells (APCs), thereby activating T cells including helper T (Th) cells type 1 and type 17 and cytotoxic T lymphocytes (CTLs). Th type 1 cells play a key role in the development of the autoimmune response. They stimulate the activity of inflammatory T cells, macrophages and natural killer (NK) cells by producing proinflammatory cytokines and stimulate B cells (B), which produces autoantibodies and inhibits the protective Th type 2 cell function. Together, these immune cells contribute to the destruction of pancreatic β-cells. Red line: Inhibition; Green line: Stimulation. GADA: Glutamic-acid-decarboxylase antibody; IA2: Islet tyrosine phosphatase 2 antibody; IAA: Insulin autoantibody; ICA: Islet cell antibody; IL: Interleukin; IFN-γ: Interferon γ; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α; Treg: Regulatory T cell; ZnT8: Zinc transporter 8 antibody.

the Juvenile Diabetes Research Foundation, the Endocrine Society and the American Diabetes Association, there are three distinct stages in T1DM[12]. Genetic predisposing factors are present from birth. The autoimmune reaction may be initiated in genetically susceptible individuals by environmental risk factors, which are not well understood[13]. People with a first- or second-degree relative with T1DM have a 15 times greater risk of developing the disease compared to the general population [14] (Figure 2).

Stage 1 is the critical point of no return since eventually the affected individuals will develop clinical diabetes. It is characterized by the presence of immune markers, two or more of the T1DM-associated islet antibodies, such as islet cell antibodies, GADA, Islet tyrosine phosphatase 2 antibodies and zinc transporter 8 antibodies, normoglycemia and absence of diabetic symptoms. In stage 2, the β-cell volume is critically decreased, and metabolic markers become detectable in asymptomatic patients. These individuals, besides being antibody positive, display impaired fasting glycemia, impaired glucose tolerance, abnormal oral glucose tolerance test or glycated hemoglobin (HbA_{1C}) ≥ 5.7%. Stage 3 represents the phase of clinical diagnosis and the manifestation of typical diabetic symptoms such as polyuria, polydipsia, weight loss, fatigue, diabetic ketoacidosis, *etc*[12]. Over time, most of the residual β cells are lost. However, sensitive C-peptide measurements have shown that 30%-80% of patients with long–standing T1DM are insulin microsecretors. This means that these patients have detectable stimulated C-peptide value of < 30 pmol/L (< 0.09 ng/mL)[15], an important consideration in therapeutic approaches targeting β-cell survival[16]. Shield *et al*[17] identified two clear phases of C-peptide decline after the diagnosis of T1DM: an exponential fall in the first 7 years (-47%/year) followed by a stable phase (-0.1%/year) (Figure 2).

Age has a major influence on the rate of disease progression. In children, the clinical stage develops more rapidly, and β-cell loss is more pronounced compared to adults. About 6 years to 9 years after the diagnosis of T1DM, 20% of those diagnosed in childhood and 60% of those diagnosed in adulthood had detectable C-peptide secretion, an indicator of endogenous insulin production[18]. In addition, the autoantibody titer and profile are also a determinant of β-cell loss. Most people will not develop diabetes if they have a single autoantibody. In contrast, the more autoantibodies a person has and the higher their serum concentration is, the rate of disease progression is greater[19]. Lately, stage-specific therapies have been the focus of clinical trials for modifying disease progression[13,19].



DOI: 10.4239/wjd.v13.i10.835

Figure 2 Stages of type 1 diabetes mellitus and options of therapeutic interventions with proven therapeutic benefits in phase II and III clinical trials. The interaction between genetic predisposing factors, which are present from birth, and environmental factors trigger the autoreactive process. Stage 1 is characterized by individuals who exhibit at least two of the type 1 diabetes mellitus (T1DM)-associated antibodies (glutamic-acid-decarboxylase-, islet tyrosine phosphatase 2-, islet cell- and zinc transporter 8 antibodies). Stage 2 is characterized by dysglycemia due to reduced β-cell function. Stage 3 represents the onset of clinical T1DM and usually but not always the manifestation of typical symptoms. The application of the previously proposed staging system[12] in clinical trials, which even recognizes the earliest stages of T1DM, can improve the research and development of novel therapies that might delay/prevent the onset of the disease. IL: Interleukin; IFG: Impaired fasting glycemia; IGT: Impaired glucose tolerance; HBA_{1C}: Glycated hemoglobin. Figure adapted from Insel *et al*[12].

DISEASE-MODIFYING THERAPEUTIC OPTIONS

Groundbreaking studies with cyclosporin A in the 1980s showed that the disease course of T1DM can be altered with immune therapy[20] and gave rise to research in the field of definitive treatment of T1DM. Despite extensive efforts so far, immune-altering therapies could not reach approval for routine clinical use for several reasons. Some agents such as cyclosporin A have many side effects and lack long-term efficacy[21]. Other options with a more favorable tolerance profile such as the adjuvant-formulated GAD-alum vaccine, which incorporated recombinant human glutamic acid decarboxylase, had no effect on disease progression[22]. The recent development of more targeted immunotherapies, the advances in regenerative therapies and lessons learned about β-cell survival from type 2 diabetes mellitus studies gave rise to more promising therapeutic options. Of the immunotherapy agents, teplizumab has come closest to achieving success. In July 2021, the United States Food and Drug Administration considered the use of teplizumab in high-risk individuals but deemed further studies necessary before granting approval[23].

B-cell targeting agents

Even though T1DM is mainly a T cell-mediated autoimmune disease, antigen-presenting B lymphocytes can also play a pathogenic role by activating T-lymphocytes and triggering the autoimmune destruction of β cells. In animal models and triggered human studies, T1DM anti-B lymphocyte therapies have been shown to be effective[24].

Rituximab: Rituximab is an anti-CD20 monoclonal antibody known to cause B-cell depletion. It is widely used in clinical practice in malignant hematological diseases such as non-Hodgkins lymphomas and chronic lymphocytic leukemias. In a placebo controlled randomized trial, including 87 recent onset T1DM patients, it has been shown that rituximab treatment was associated with slower progression of β -cell dysfunction and better metabolic control during the 12-mo long study period[25]. Insulin dose requirements decreased during the study. However, none of the patients were able to become insulin

free. Extensive follow-up of the patients showed a constant decline in C-peptide production. It suggests that B-cell depletion by its own is not sufficient in restoring β-cell tolerance in the long run and does not fundamentally alter the course of overt T1DM[26].

It has been reported that rituximab can suppress insulin autoantibodies, but no such effect could be found in the case of GADA, islet tyrosine phosphatase 2 antibody and zinc transporter 8 antibody[27]. Compared to placebo controls, rituximab-treated T1DM patients, whose C-peptide response was significant, have shown increased proliferative responses to islet, neuronal and disease-relevant environmental antigens ultimately resulting in increasing insulin secretory function[28]. Moreover, the combination of rituximab with $CD4^+$ $CD25^{high}$ $CD127^-$ T regulatory cells[29] or therapy targeting $CD4^+$ T cells[30] can further improve treatment efficacy. One side effect of rituximab, reported by the study of Kroll *et al*[31], might be the reactivation of some asymptomatic polyomavirus infections. Rituximab is currently being tested in earlier stages of T1DM (NCT03929601[32]).

T-cell targeting agents

T-cell co-stimulation inhibition: Abatacept is a cytotoxic T lymphocyte-associated antigen 4 immunoglobulin fusion protein designed to selectively bind to CD80/86 to inhibit the early activation and proliferation of naïve T lymphocytes. Since effector memory T cells are less dependent on CD28 costimulation, abatacept is a more selective way to inhibit T cell activation compared to general immunosuppressants. In a phase II placebo-controlled study, Orban *et al*[33] investigated the effect of abatacept in a population with recent onset T1DM. Patients in the treatment group received a monthly dose of 10 mg/kg intravenous abatacept for 2 years. The authors found that cytotoxic T lymphocyte-associated antigen 4 inhibition slowed the decline of β-cell function during the 2 years of the treatment and had a beneficial effect during the 1-year follow-up period without active treatment[34]. However, the observed positive effect was only temporary and declined over time. Furthermore, it was found that abatacept does not change immunogenicity of other vaccines in T1DM patients[35], but different follicular Th and central memory $CD4^+$ T cell phenotypes might affect the efficacy of the treatment[36, 37]. There are still ongoing clinical trials (*e.g.*, NCT01773707[33], NCT04118153[38] and NCT03929601 [32]) investigating whether abatacept may have a more potent impact on the disease if administered at earlier stages (*e.g.*, at stage 2) of T1DM pathogenesis.

Anti-CD3 therapy: So far the most promising therapeutic target in modifying the course of T1DM is the ε chain of the CD3 receptor on the T cell surface, previously known as muromonab-CD3 (trade name: Orthoclone OKT3)[39]. Animal studies have shown that anti-CD3 therapy can induce diabetes remission in the models of T1DM[40]. This main effect is associated by the induction of Treg cells and immunosuppressive cytokines such as transforming growth factor β[41]. One of the first human trials reported significantly improved C-peptide response and other clinical parameters after a single shot of hOKT3gamma1(Ala-Ala)[42].

Teplizumab is a humanized anti-CD3 monoclonal antibody, which has been shown to be the most potent agent in slowing the progression of T1DM. In a series of human clinical trials, it was demonstrated that the treatment of teplizumab was a potent way to delay the decline of C-peptide production[43-45] and can help to preserve β-cell function[46], and its effect can be sustained by an average of 15.9 mo in T1DM[47]. Furthermore, the Protegé[48,49] study in which 516 T1DM patients were enrolled and treated with teplizumab has demonstrated that anti-CD3 therapy delayed the decline of insulin secretion and induced disease regression, and 5% of the patients became insulin independent. Even though teplizumab has shown promising results in preventing disease progression, it must be noted that significant metabolic benefits such as a significant reduction in HbA_{1C} could not be demonstrated.

These promising results led to further trials to evaluate the effect of anti-CD3 therapy in T1DM prevention. The recently published results of a phase III follow-up trial including non-diabetic patients at high risk of developing T1DM, defined as having impaired glucose tolerance and at least two diabetes-specific autoantibodies, demonstrated the efficacy of teplizumab in delaying the onset of T1DM by 48.4 mo compared to the placebo group (24.4 mo)[50]. C-peptide levels of those who responded to treatment remained significantly better even after a 7-year follow-up period[51]. Furthermore, clinical responders to teplizumab therapy have shown significant reduction in circulating $CD4^+$ effector memory T cells and decreased activation and regulatory gene expression in circulating $CD8^+$ central memory T cells[52]. Currently, studies are running to investigate teplizumab in at-risk individuals[53] and recent-onset T1DM patients[54,55].

Otelixizumab is another humanized anti-CD3 antibody that has been evaluated both in the treatment of overt[56] and new-onset[57,58] T1DM. Similarly to teplizumab, a 6 d treatment of otelixizumab preserved residual β-cell function for at least 18 mo in 40 patients with recent-onset T1DM. The protective effect of otelixizumab treatment appears to be dose dependent as studies attempting to lower adverse reactions by administering lower doses could not demonstrate a benefit in C-peptide preservation[59,60]. The protective effect of otelixizumab showed the highest benefit in insulin autoantibody-positive T1DM patients[61].

Anti-CD3 therapies have been overall well tolerated among patients. Adverse reactions that were significantly more prevalent in the treatment group included: vomiting, rash, chills, cytokine release syndrome, Epstein-Barr virus reactivation[57,62] and headache. Recently, a subcutaneous formulation was also introduced[63], which significantly reduced such undesirable effects. Adverse reactions were mostly mild to moderate and self-limited; 9% of patients were not able to complete all drug doses compared to a 2% dropout rate in the placebo group. The most common cause for treatment cessation was lymphopenia, neutropenia, elevated liver enzymes and reduced platelet counts.

Low dose antithymocyte globulin: Antithymocyte globulin (ATG) is a polyclonal immunoglobulin G antibody against multiple human T cell antigens and their precursors. Only a limited number of studies are available, and their results are somewhat controversial: 6.5 mg/kg ATG alone could not preserve β-cell function, but C-peptide secretion was preserved in older participants suggesting a possible age-specific action[64,65]. In contrast, low dose ATG treatment (2.5 mg/kg administered as 0.5 mg/kg on day 1 and 2 mg/kg on day 2) in combination with pegylated granulocyte colony-stimulating factor acts by decreasing the number of activated effector T cells while relatively preserving Treg cells. T1DM patients with a diabetes onset between 4 mo and 2 years receiving low dose ATG + granulocyte colony-stimulating factor have shown a benefit in disease progression[66,67]. Patients in the treatment group have had higher C-peptide production after a mixed meal test, and lower HbA_{1C} after 6 mo was also recorded in the treatment group compared to the placebo group.

A more recent study published by the same group indicated that low-dose ATG monotherapy without granulocyte colony-stimulating factor can delay the decline of C-peptide, can reduce the HbA_{1C} level and affect T cell phenotypes in new-onset T1DM[68,69]. The ongoing follow-up of this study is in progress along with two additional studies[70,71], and their results will help further evaluate the potential benefits of low-dose ATG and its therapeutic potential for preventing T1DM.

Anti-IL-21 and liraglutide: A new strategy to modify the disease course in T1DM is using a drug combination that not only halts or delays the progressive autoimmune process but aims at preserving and improving residual β-cell function. This approach may have the advantage over previous monotherapies in achieving disease modification with milder immunomodulation in a safer, more sustainable way. IL-21 plays a key role in the pathomechanism of T1DM by activating and leading $CD8^+$ T lymphocytes from lymph nodes and the exocrine pancreas to the pancreatic islets eventually leading to β-cell destruction[72,73]. Based on these findings, IL-21 inhibition has emerged as a potential disease-modifying target in preventing T1DM. 35-Liraglutide, a glucagon like peptide-1 analog that has routinely been used in type 2 diabetes therapy, has been proven to increase β-cell survival[74] and can improve glucose dependent insulin secretion not only in type 2 diabetes but in T1DM as well[75,76].

In a recent phase II clinical trial the effect of liraglutide and IL-21 inhibition was evaluated in 308 T1DM patients with recent onset disease and residual β-cell function[77]. The combination treatment was effective to preserve both fasting and postprandial endogenous insulin secretion resulting in a nonsignificant decrease in the number of hypoglycemic events and level of HbA_{1C} for 52 wk. During the follow-up period the combination treatment was considered safe, and there were no safety concerns raised. The study included a 26 wk off-drug observation period during which the effect of the treatment deteriorated rapidly, suggesting the need for continued treatment. Overall, this combination treatment seems to be a promising candidate for further evaluations in a phase III clinical trial.

MESENCHYMAL STEM CELL THERAPY IN T1DM

Characteristics of mesenchymal stem cells

Over the past two decades, stem cell transplantation has received increased attention in clinical trials as a promising therapy within regenerative medicine for T1DM. While the treatment of T1DM with hematopoietic stem cells was more typical in the 2000s and first half of the 2010s[78], the most recent studies focus more on the treatment with mesenchymal stem cells (MSCs)[13]. This modern approach to treat T1DM has several advantages over previous treatment options. MSC transplantation is hypo-immunogenic because the cells do not express costimulatory antigens (CD80, CD86, CD40, CD40L *etc*) nor major histocompatibility complex II and major histocompatibility complex I. MSCs allow both autologous and allogeneic transplantation, even without conditioning treatment[79].

MSCs can be easily cultured *in vitro* due to their high dividing capacity, and they can be isolated from many adult and perinatal sources (*e.g.*, bone marrow, adipose tissue, peripheral blood, dental pulp, skeletal muscle, liver, lung, umbilical cord blood, Wharton's jelly and placenta)[80]. Of these, the umbilical cord and its derivatives stand out as they can be obtained non-invasively, are considered as 'medical waste' and have an exceptional differentiation capacity towards insulin-secreting cells[81]. Unlike embryonic stem cell therapy, the use of these tissue sources does not raise special ethical issues.

In addition, MSCs have no known tumorigenic effect, whereas embryonic stem cells can form teratomas and teratocarcinomas *in vivo*[82]. However, tumorigenesis cannot be completely ruled out as a possible adverse effect: MSCs may be a direct source of malignant cells, may maintain various cancer processes (*e.g.*, breast and colon cancer) through paracrine factor secretion or may enhance tumor

growth and progression through their immunosuppressive effects[83-85]. However, to the best of our knowledge, no similar side effects have been reported in clinical trials using MSCs[86].

MSCs are capable of generating tissue types of mesodermal origin, such as musculoskeletal, cartilaginous and adipose tissue, and may cross the boundaries of germ layers and transdifferentiate into ectodermal neurons or even endodermal islet cells[87,88]. Low amounts of MSCs in the target tissue do not explain regeneration or even wound healing. However, *in vivo* experiences show that MSCs have other, more pronounced therapeutic effects, such as remodeling of the diabetic microenvironment[89, 90]. It should be noted that in systemic administration, MSCs are entrapped in capillaries, especially in the lungs, reducing the number of migrating cells towards the target tissue, suggesting that better outcome could be obtained through local injection[91].

Immunoregulatory function of MSCs in T1DM

The strong immunoregulatory function of MSCs plays a key role in the regeneration of β cells. This protective effect in T1DM is due to secretion of soluble factors and cell-cell interactions. Insulin deficiency and irreversible β-cell destruction are the consequences of the autoimmune reaction in T1DM, and MSCs are able to intervene at several points in this process, modulating immune cells. MSC transplantation with its paracrine effects, due to the production of cytokines, chemokines and growth factors, can affect the local environment, inhibit apoptosis and induce proliferation. The identified bioactive factors are: IL-6, IL-8, transforming growth factor β, vascular endothelial growth factor, hepatocyte growth factor and nitric oxide[13].

Two types of MSCs are known: proinflammatory MSCs (MSC1) and anti-inflammatory MSCs (MSC2). The type of polarization depends on the inflammatory milieu. In the absence of an inflammatory environment MSCs adopt a proinflammatory phenotype and amplify T cell responses. Conversely, in an inflammatory environment (high interferon-γ and tumor necrosis factor α levels), MSCs may adopt an immunosuppressive phenotype and suppress T cell proliferation *via* secreted soluble factors[92]. MSCs have a regulatory function against effector T cells. In the pathogenesis of T1DM, Th1 cells are the main effector cells, and Th2 cells have been shown to be protective.

Beneficial effects of MSCs in diabetes can be attributed to: (1) Secreted IL-4; (2) Altered Th1/Th2 ratio with a Th1 to Th2 shift; and (3) Promoted maturation of naïve T cells towards Th2[93]. Furthermore, MSCs can directly and indirectly inhibit through several pathways: (1) Th17 cell development and thus IL-17 production; (2) CTL function and thus Fas-mediated β-cell apoptosis; and (3) Both maturation and activation of antigen-presenting cells, principally dendritic cells, by secreting for example prostaglandin E2, IL-6 and macrophage colony-stimulating factor[13,94,95].

Two types of macrophages are known: M1 and M2 producing proinflammatory and anti-inflammatory cytokines, respectively. MSCs can modulate the phenotype shift, causing an M1 to M2 shift[96]. Treg cells are components of MSC-induced indirect immunosuppression. *In vivo* and *in vitro*, MSCs have been shown to enhance Treg proliferation through cell-cell interaction[13]. By producing IL-10 and transforming growth factor β, Treg cells downregulate Th1- and Th17-mediated inflammatory response and the cytotoxicity of CTLs, thereby leading to immune tolerance in the organism[97]. These mechanisms can contribute to both amelioration of auto-reactivity and of β-cell death (Figure 3).

Clinical application of MSC therapy for the treatment of T1DM

In recent years, MSCs have attracted the attention of many researchers and clinicians as a result of encouraging preclinical animal data in T1DM. The most important advantages are: (1) Wide range of sources; (2) Self-renewal capacity; (3) Multidifferentiation capacity; and (4) Strong immunomodulatory potential. MSCs are also immunoprivileged, well-tolerated and safe[98]. The clinical studies vary in MSC origin, dose, route of transplantation, administration frequency and in eligible patients' characteristics (Table 1).

Hu *et al*[99] studied the long-term effects of Wharton's jelly-derived MSC in newly diagnosed T1DM patients. Group 1 was treated with parenteral solution of Wharton's jelly-derived MSCs by intravenous delivery, while the control group received normal saline. In the treatment group HbA_{1C} reached its lowest value after half a year and then began to fluctuate. Fasting C-peptide showed a progressive increase, reaching its maximum after 1 year; 3/15 patients were insulin-free, and 8 had their insulin dose halved after 2 years. As the study follow-up period lasted 2 years, exceeding the average 1.5 year honeymoon period, the therapeutic effect was due to MSCs[99]. This was one of the first studies to prove the safety and effectiveness of MSCs.

Thakkar *et al*[100,101] used the combination of adipose-derived insulin-secreting mesenchymal stem cells and bone marrow-derived (BM-) hematopoietic stem cells, comparing autologous (group 1) and allogeneic (group 2) stem cells. The study procedure was as follows: Resection of adipose tissue from the abdominal wall, collected in proliferation medium, bone marrow aspiration, conditioning treatment with bortezomib, methylprednisolone, ATG, and finally injection of the mixed inoculum. Autologous stem cell therapy offered better long-term control of hyperglycemia, but the two groups fairly differed in baseline mean C-peptide levels[100]. Although the two treatment methods showed significant differences in carbohydrate metabolism, the results before and after stem cell therapy were not statistically analyzed within the groups, thus lacking conclusive information about the efficacy of MSCs. As an early-result, the group reported preliminary data of 10 patients[101]. After an approximately 3-year

Table 1 Summary of clinical trials using mesenchymal stem cells in type 1 diabetes mellitus

Ref.	Patient characteristics	Treatment	Therapeutic outcomes
Wu *et al*[105], 2022[1]	*n* = 14; aged 27-47 yr	Intrapancreatic: Allogeneic UC-MSC + autologous BM-MNC	Insulin independence: No
China, 8 yr	Duration of T1DM: 10-24 yr		Insulin requirement: Improvement at 1 yr but no difference at 8 yr
			FCP and HbA_{1C}: Significant improvement
			Significantly lower occurrence of diabetic complications
Izadi *et al*[108], 2022	*n* = 20; aged 8-40 yr	BM-MSC	Insulin independence: No
Iran, 12 mo	Duration of T1DM: < 1 yr (*n* = 11) and > 1 yr (*n* = 9)		Insulin requirement, FCP, HbA_{1C}: Significant improvement
	FCP (*n* = 11): 0.92 ± 0.57 ng/mL		Number of hypoglycemic events decreased
			Patients with early onset of T1DM benefit more
			Adverse effects: Possible mild injection site reactions
Lu *et al*[106], 2021	*n* = 27; aged 8-55 yr	IV (2x): Allogeneic UC-MSC	Insulin independence: 3 subjects
China, 12 mo	Median duration of T1DM: 2.3 mo		Insulin requirement, HbA_{1C}: No improvement
	FCP: 100 pmol/L (0.3 ng/mL)		SCP: improved in adult-onset T1DM subgroup
			Adverse effects: Mild fever
Dantas *et al*[103], 2021[2]	*N* = 7; Aged 16-35 yr	Allogenic AD-MSC + 2000 UI/d cholecalciferol	Insulin independence: 1 subject
Brazil, 6 mo	Duration of T1DM: ≤ 4 mo		Insulin requirement: Stable at 6 mo
	FCP: 0.80 ± 0.38 ng/dL		FCP and HbA_{1C}: Significant improvement
			Adverse effects: Transient headache, mild local reactions, immediate tachycardia, thrombophlebitis + other mild effects
Araujo *et al*[102], 2020	*n* = 8; aged 16-28 yr	Allogenic AD-MSC + 2000 UI/d cholecalciferol	Insulin independence: 2 subjects
Brazil, 3 mo	Duration of T1DM: ≤ 4 mo		Insulin requirement, HbA_{1C}: Decreased significantly at 3 mo
			FCP: Only initial improvements, with the same results at the 3-mo visit
			Adverse effects: Transient headache, mild local reactions, immediate tachycardia, thrombophlebitis + other mild effects
Cai *et al*[104], 2016	*n* = 21; aged 18-10 yr	Intra-pancreatic: Allogeneic UC-MSC + autologous BM-MNC	Insulin independence: No
China, 12 mo	Duration of T1DM: 2-16 yr		Insulin requirement, HbA_{1C}: Decreased significantly
	FCP: < 0.1 pmol/mL (< 0.3 ng/mL)		FCP: Markedly increased
			Adverse effects: Transient abdominal pain, bleeding
Carlsson *et al*[107], 2015	*n* = 9; aged 18-40 yr	IV: Autologous BM-MSC	Insulin independence: No
Sweden, 12 mo	Duration of T1DM: < 3 wk		Insulin requirement, HbA_{1C}, SCP: No significant improvement
	SCP: > 0.1 nmol/L (> 0.3 ng/mL)		Adverse effects: No
Thakkar *et al*[100], 2015	*n* = 20; aged 8-45 yr	Into portal + thymic circulation and subcutaneous tissue:	Insulin independence: No
India, 24 mo	Duration of T1DM: > 12 mo		Insulin requirement: Decreased

	2 groups with a mean C-peptide:	Group 1: Autologous IS-AD-MSC+ HSC	HbA_{1C}, C-peptide: Sustained improvement
	Group 1: 0.22 ng/mL	Group 2: Allogeneic IS-AD-MSC+ HSC	Adverse effects: No
	Group 2: 0.028 ng/mL		
Dave *et al*[101], 2015[3]	*n* = 10; aged 9-29 yr	Into portal + thymic circulation and subcutaneous tissue: autologous IS-AD-MSC+ HSC	Insulin independence: No
India, 27 mo	Duration of T1DM: 2-15 yr		Insulin requirement: Decreased
	Pre-IV C-peptide: 0.22 ng/mL		HbA_{1C}, C-peptide: Sustained improvement + significantly lower GADA levels
			Adverse effects: No
Hu *et al*[99], 2013	*n* = 15; aged < 25 yr	IV (2x): Allogeneic WJ-MSC	Insulin independence: 3 subjects
China, 24 mo	Duration of T1DM: < 6 mo	Control group: normal saline	Insulin requirement: 8 patients more than 50% reduction
	C-peptide: ≥ 0.3 ng/mL		HbA1C: Significantly decreased; FCP: Significantly increased
			Adverse effects: No

[1]Follow-up trial to Cai *et al*[104], 2016.
[2]Extension trial to Araujo *et al*[102], 2020.
[3]Preliminary data of Thakkar *et al*[100], 2015.
AD: Adipose-derived; BM: Bone marrow-derived; FCP: Fasting C-peptide; GADA: Glutamic-acid-decarboxylase antibody; HbA_{1C}: Glycated hemoglobin; HSC: Hematopoietic stem cell; IS-AD: Adipose-derived insulin-secreting; IV: Intravenous; MNC: Mononuclear cell; MSC: Mesenchymal stem cell; SCP: Stimulated C-peptide; T1DM: Type 1 diabetes mellitus; UC: Umbilical cord-derived; WJ: Wharton's jelly-derived.

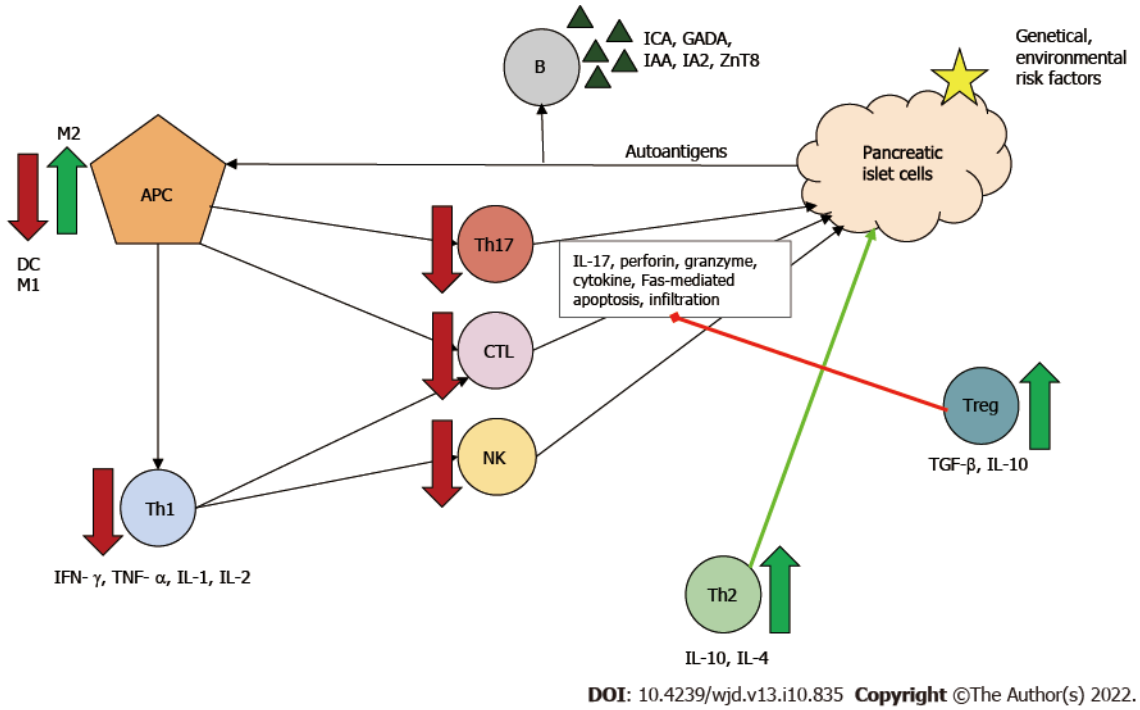


Figure 3 Immunoregulatory function of mesenchymal stem cells in type 1 diabetes mellitus. The effects of mesenchymal stem cells ultimately result in downregulation in the proliferation and function of dendritic cells, cytotoxic T lymphocytes (CTLs), helper T (Th) type 1 and type 17 cells, natural killer (NK) cells and type 1 macrophages (M1). Meanwhile, mesenchymal stem cells increase the number of type 2 macrophages (M2) and regulatory T cells (Tregs), which can inhibit effector T cells and stimulate Th type 2 protective cells. Red arrow/line: Downregulation/inhibition; Green arrow/line: Stimulation. APC: Antigen-presenting cell; B: B cell; DC: Dendritic cell; GADA: Glutamic-acid-decarboxylase antibody; IA2: Islet tyrosine phosphatase 2 antibody; IAA: Insulin autoantibody; ICA: Islet cell antibody; IL: Interleukin; IFN-γ: Interferon γ; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α; ZnT8: Zinc transporter 8 antibody.

follow-up, increased C-peptide secretion, decreased exogenous insulin requirement, improved HbA_{1C} and significantly lower GADA levels have been found.

Similar results were obtained in the studies of Araujo *et al*[102] and Dantas *et al*[103], where a single dose of adipose-derived MSC infusion were combined with daily 2000 IU vitamin D_3 supplementation. Compared to control subjects on traditional treatment, improved HbA_{1C} levels and reduced insulin doses have been found 3-mo after MSC infusion[102], while basal C-peptide levels remained the same at first but significantly improved for the 6-mo follow-up measurement[102,103]. It has to be mentioned though that most patients reported transient headache and local reactions, and further mild but resolving adverse events were also reported by a significant amount of the study population. Although the results of this study further strengthened the efficacy and safety of adipose-derived MSCs, all positive effects were somewhat overshadowed by the significant number of adverse events.

Cai *et al*[104] investigated the safety and efficacy of umbilical cord-derived MSC (UC-MSC) and autologous BM-mononuclear cell cotransplantation in adult patients. The treatment group received octreotide as a prophylaxis, followed by stem cell infusion into the dorsal pancreatic artery. After 1 year, the C-peptide area under the curve during a 3-h oral glucose tolerance test increased by 105.7% in 20 of 21 responders compared to a 7.7% decrease in the control group showing the robust effect of the treatment against disease progression. Further importance of this trial was that immunological parameters were also assessed. GADA positivity remained unchanged, while IL-10 levels increased, and interferon-γ levels and adenosine triphosphate levels in $CD4^+$ T cells decreased. While the effect of MSCs may be less pronounced in this study due to reduced inflammatory signals in long-standing disease[104], it has to be mentioned that the long-term follow-up analysis of the study population have shown a significantly decreased occurrence of various diabetic complications. Furthermore, the UC-MSC treated patients still had clinically better HbA_{1C} and C-peptide levels, 8 years after the UC-MSC treatment, but the initial difference in insulin requirement leveled off[105]. The combined results of the original and follow-up study[104,105] indicate that UC-MSCs are good candidates for slowing down the progression of T1DM.

Lu *et al*[106] assessed the repeated transplantation of allogeneic UC-MSC in T1DM. The primary efficacy endpoint was clinical remission, defined as a 10% increase from baseline in the levels of fasting and/or postprandial C-peptide. After 1 year, 11 out of 27 in the UC-MSC-treated group maintained clinical remission, whereas only 3 out of 26 in the control group maintained clinical remission. The UC-MSC-treated group showed a decreasing trend in fasting and postprandial C-peptide. Three UC-MSC-treated adults became insulin independent and started using insulin again in 3-12 mo. Among adult-onset T1DM (≥ 18 years of age), UC-MSC treatment showed a protective effect on β-cell function but failed to be protective in juveniles. Three recipients had mild fever after UC-MSC infusion; all of them recovered within 24 h[106]. It seems UC-MSC therapy might be more beneficial for patients with adult-onset T1DM.

Carlsson *et al*[107] tested the efficacy of autologous BM-MSCs in newly diagnosed T1DM patients. Stems cells were harvested from the aspiration of the iliac crest and subsequently administered to the MSC-treated group as an intravenous infusion without premedication. HbA_{1C}, fasting C-peptide and insulin requirement were not significantly different compared to the control group[107]. In contrast, Izadi *et al*[108] found improved HbA_{1C} and C-peptide levels, a reduced number of hypoglycemic events and increased anti-inflammatory patterns. Furthermore, early BM-MSC transplantation (< 1 year after disease onset) further improved HbA_{1C} levels and C-peptide levels compared to those who received the transplantation > 1 year after disease onset[108], similar to that of UC-MSC.

Summarizing the available clinical study results of the stem cell therapies, the results about BM-MSC and adipose-derived MSC are more controversial, suggesting that these two therapies may be less effective than UC-MSC therapy in T1DM. It should be noted, however, that based on the results of the studies so far it is recommended to apply these treatments as early as possible. The earlier these treatments are introduced, the greater the preservation of the remaining β cells, thereby the reduction of external insulin requirement and the development of long-term complications can be elongated. Adipose-derived MSCs and UC-MSCs are currently under further investigated in NCT05308836[109] and NCT04061746[110], respectively.

CONCLUSION

In the management of T1DM the focus remains on the challenges of glycemic control and long-term complications, which could not been fully overcome by new technological advances. Recently, there has been a paradigm change in the treatment of T1DM. The goal now is to cure rather than identify a lifelong 'symptomatic treatment' with insulin supplementation. The crucial future may lie in disease-modifying therapeutic options, which could be used to preserve β cells in the presymptomatic phase of the disease and to cease the destructive autoimmune process as well as to regenerate β-cell function in the clinical phase.

Immunotherapy appears to be a promising disease-modifying therapy in T1DM. Different agents have the potential to target the major autoreactive immune pathways leading to T1DM. Therapies interfering with T cell activation seem to be the most favorable. Regenerative therapy is developing parallel with immunotherapy. MSC therapy stands out from other cell therapies. It is safe, with its

beneficial effects due to immune regulation. However, the clinical trials are limited in their conclusions due to the small patient numbers and short follow-up times. Standardized stem cell processing, transplantation protocols and dosage will be essential for future randomized, double-blinded clinical trials with large patient cohorts. Combining disease-modifying therapies with glucagon like peptide-1 analogues seem to increase efficacy and increase tolerability of interventions.

So far, neither immunotherapies nor stem cell therapies, when used alone, have had ultimate successes in altering T1DM disease course. Their common disadvantage is that their short-term therapy effects are transient. The future for disease-modifying therapies might be the individualized, long-term multimodal approach combining immune, incretin based and regenerative therapeutic options potentially by identifying biomarkers of responders for it to be used in routine clinical treatment.

ACKNOWLEDGEMENTS

We are grateful to Professor Lindner E (Memphis University) for English proofreading.

FOOTNOTES

Author contributions: Nagy G and Szekely TE wrote the manuscript with the support of Somogyi A, Herold M and Herold Z.

Conflict-of-interest statement: All authors report no relevant conflict of interest for this article.

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S-Editor: Wu YXJ
L-Editor: Filipodia
P-Editor: Wu YXJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 851-860

DOI: 10.4239/wjd.v13.i10.851

ISSN 1948-9358 (online)

MINIREVIEWS

Advances in traditional Chinese medicine as adjuvant therapy for diabetic foot

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): E

P-Reviewer: Li MR, China; Liu YJ, China; Miranda C, Italy

Received: June 16, 2022
Peer-review started: June 16, 2022
First decision: July 14, 2022
Revised: July 31, 2022
Accepted: August 26, 2022
Article in press: August 26, 2022
Published online: October 15, 2022

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Abstract

Diabetes mellitus (DM) is a complex disease that often causes multiple systemic complications that have become a major international public health problem. Diabetic foot (DF) is one of the severe and frequent chronic complications of DM due to vascular lesions and neuropathy. DF ulcers (DFU) affect approximately 15% of people with DM and are the leading cause of death and disability. The prevalence and recurrence of DF are worrisome, and morbidity and mortality are also on the rise, which poses a substantial socioeconomic burden. Treating DF is difficult for clinicians and requires multidisciplinary cooperation, combining local and systemic therapy to reduce amputation and case-fatality rates. Traditional Chinese Medicine (TCM) has received extensive attention due to noticeable therapeutic effects and few adverse reactions. In recent years, research on DF treatment by TCM has been increasing, and further progress has been made. TCM includes oral medication, injectable preparations, and adjuvant therapy. This article reviews the relevant research on TCM-related adjuvant therapy for DF. We describe current progress in TCM in terms of external application, acupuncture, massage, acupoint injection, foot bath, fumigation, and moxibustion, as well as the mechanisms involved.

Key Words: Diabetes Mellitus; Diabetic foot; Foot ulcers; Traditional Chinese medicine; Wound healing

Core Tip: Diabetic foot (DF) is a serious complication of diabetes and has become a major global health problem. Despite the emergence of many new therapies, amputation and mortality rates remain high. Traditional Chinese Medicine (TCM) has proved effective for various diseases, and more studies have observed its value of Traditional as adjuvant therapy. We review the role of TCM adjuvant therapy for DF, including external application, acupuncture, moxibustion, massage, acupoint injection, foot bath, and fumigation.

Citation: Liu FS, Li Y, Guo XS, Liu RC, Zhang HY, Li Z. Advances in traditional Chinese medicine as adjuvant therapy for diabetic foot. *World J Diabetes* 2022; 13(10): 851-860
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/851.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.851

INTRODUCTION

Diabetes mellitus (DM) is a complicated metabolic disorder caused by various pathogenic factors, and the main clinical feature is abnormally elevated blood glucose[1]. The American Diabetes Association divides diabetes into types 1 diabetes, type 2 diabetes, specific types of diabetes due to other causes, and gestational DM[2]. The prevalence of DM in virtually all world regions has increased significantly in recent decades. The estimated number of people with DM worldwide was 451 million in 2017. Approximately 1 in 11 adults has DM, and 90% of them have type 2 diabetes; that number is expected to increase to 693 million by 2045[3,4]. The main goal of therapy for type 2 diabetes is to prevent or delay complications and maintain quality of life[5]. There are many complications of DM, such as cardiovascular disorders[6], end-stage renal disease[7], retinopathy[8], neuropathy[9], mental illness[10], muscle atrophy[11], adhesive capsulitis[12] and even joint stiffness following surgery[13,14]. Diabetic foot (DF) is a frequent complication of DM due to vascular and neuropathological damage and is the main reason for amputation and death[15]. About 15% of people with DM suffer from DF ulcers (DFUs), and 14%-24% of those with DFU subsequently undergo lower limb amputation, which has led to DFU being the leading cause of non-traumatic lower limb amputations[16]. The 5-year mortality after amputation is 50%-59%[17], which is higher than the 5-year pooled mortality rate for cancer, which is 31.0%[18]. The global prevalence of DFUs is 6.3%, and in North America, this figure is 13.0%[19]. Moreover, DFU has a recurrence rate of 22.1% per person per year[20]. The direct cost of DM care in the USA in 2017 was US$237 billion, of which one-third was for lower extremity complications[18]. Healthcare expenditure for DF care is even more in the UK than for breast, prostate and lung cancer combined[21]. These data prove that DF has become a serious international medical and health problem. Therefore, understanding the pathogenesis of DF and developing targeted treatment is a major concern to clinicians.

DF is prone to ulceration and infection due to neuropathic edema and occlusive arterial disease[22]. DFU is caused by various factors, including peripheral neuropathy, foot deformity and trauma, and arterial disease[23]. In addition, DFU development was linked with a previous history of DFU and the male sex[24]. The precise mechanism of the delayed healing of DFU has not been fully elucidated. Wound healing is one of the most complex processes in the human body, mainly including four phases (hemostasis, inflammation, growth, re-epithelialization, and remodeling). Each stage has no recognizable boundaries and overlaps in time and space[25]. In DFU, however, extensive defects in the healing process result in ulcer healing delay and the occurrence of a highly pro-inflammatory chronic wound[26]. The major causes may be insufficient neovascularization, neuropathy, high probability of infection, tissue hypoxia[27], and nonphysiological inflammatory response[28]. They may also include an imbalance between metabolism and nutrient transport, abnormal cellular and gene expression, excessive formation of advanced glycation end products (AEGs)[29], and high concentrations of metalloproteases[30]. Pathologically, DFU has been found to decrease endothelial progenitor cell (EPC) recruitment due to reduced NO production[31]. Deficiency of cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, keratinocyte growth factor (KGF), and platelet-derived growth factor (PDGF) are also associated with delayed DFU wound healing[29]. Furthermore, the dysfunction of the immune system in DM increases the rate of ulcer infection[32], and the frequency and severity of the infection are associated with delay and failure of the healing process [33].

Standard therapy of DFU includes decompression and ulcer protection, tissue perfusion repair, infection and metabolic control, local ulcer care, and education of patients and families[34]. TCM is the main form of ancient Asian medicine and an essential element of the Chinese health system[35], which is commonly used in clinical work in China[36]. It has accumulated a solid theoretical foundation in practice for thousands of years[37]. Chinese herbal medicine decoction is the essence of TCM, which has the characteristics of a multitarget, fewer side effects, and significant therapeutic effects[38]. With the

advent of bioinformatics, the specific mechanism of TCM has been more scientifically explained[39]. Research on TCM for chronic non-communicable diseases has recently developed rapidly[40]. In particular, studies have reported that oral administration or injection of TCM herbal-based agents as an additional treatment to conventional therapies is beneficial to DFU healing[41,42]. Meanwhile, in recent years, complementary modalities have also demonstrated therapeutic potential. These methods include external application, acupuncture, massage, acupoint injection, foot bath, fumigation, and moxibustion. Therefore, a comprehensive search was conducted in the PubMed, Web of Science, and National Knowledge Infrastructure (CNKI) to investigate the value of TCM adjuvant therapy in DF. The electronic search was performed for articles published from inception to June 20, 2022. The search terms were used individually or combined: "Traditional Chinese Medicine," "Diabetic foot," "Diabetes Foot," "Diabetic Patients with DF," "Diabetes Feet," "DF," "External application," "Dressing," "Acupuncture," "Pharmacoacupuncture," "Moxibustion," "Massage," "Acupressure," "Knead," "Acupoint injection," "Acupuncture point injection," "hydro-acupuncture," "Foot bath," "Lavipeditum," "Soak," "Medicated bath," "Fumigation." Reference lists of relevant articles were also hand searched. In addition, we made appropriate modifications according to actual requirements.

EXTERNAL APPLICATON

Using plaster or compounds of TCM for external application is an efficient and straightforward treatment method. Compound Phellodendron liquid, which consists of Forsythia, Phellodendron, Honeysuckle, Dandelion, and Centipede, is one of the TCM for external application. Network pharmacology analysis shows that it contains 36 active ingredients related to DF. Functionally, the potential mechanisms of action are mainly related to inflammatory response and growth factor activity[43]. When DFU was treated with Compound Phellodendron liquid for four weeks, the ulcer area reduction, growth factor concentration, and total effective rate in the treatment group were higher than the standard nursing group[44]. Zhong *et al*[45] prepared a mixed ultramicro powder with Angelica, Calcined Gypsum, and Caleramide as raw materials, which promoted wound healing in DFU by accelerating wound closure and epithelialization, and inducing angiogenesis. Similarly, external application of the Chinese herbal medicine compound Tangzu Yuyang Ointment combined with standard wound treatment improved the rate of DFU healing. However, the healing time appeared to be prolonged[46].

In rats with DFU, Chinese medicine ulcer oi (Cortex Phellodendri and Angelica japonica as the main ingredients) upregulated the expression of VEGF and PDGF and downregulated protein tyrosine phosphatase 1B and AGEs in the wound tissue[47]. This indicated that Chinese medicine ulcer oil reduced local wound inflammation, promoted angiogenesis, and facilitated ulcer healing. Shixiang ointment promoted angiogenesis and accelerated ulcer healing by reducing AGEs and their receptors, activating nuclear factor kB p65, and upregulating VEGF, CD34, and endothelial NO synthase in the granulation tissue of DFU rats[48]. Wan *et al*[49] proved that San-huang-sheng-fu oil reduced cyclooxygenase-2 and upregulated VEGF and improved the decrease in plantar temperature and pain sensation in rats caused by the diabetic peripheral circulatory disorder. Similarly, another agent, Jing Wan Hong Ointment, elevated PDGF expression in a DFU murine model, enabling almost complete ulcer healing *via* retarding inflammation and promoting cell proliferation and angiogenesis[50].

The common factors involved in the beneficial effects of external application of TCM in DF treatment rely on VEGF and PDGF to promote angiogenesis, cell proliferation, and inhibition of local inflammatory response.

ACUPUNCTURE

Acupuncture is essential to TCM and has been used for thousands of years against many disorders, including vascular diseases. There are many modalities of acupuncture, such as encircling needling, Bangci (focal center-side needling), auricular acupuncture, pestle needling therapy, and traditional acupuncture[51]. After auricular acupuncture treatment in type 2 DM patients, the blood flow of the lower extremities is improved, and the temperature of the soles of the feet increases, showing a preventive effect against DF[52]. Pestle needling therapy can decrease the foot vibration perception threshold and improve the sensory nerve function of the foot and the quality of life in high-risk DF[53].

Wei *et al*[54] compared the efficacy of encircling needling and Bangci (focal center-side needling) in wound healing of mice with DM. Both promoted skin wound healing by increasing local blood perfusion, and the therapeutic effect of encircling needling was better than Bangci. Mechanistically, acupuncture may reduce the protein levels of proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β and increase neovascularization and fibroblasts in the wound[55]. These outcomes indicate that acupuncture can promote wound healing by reducing inflammation, promoting cell proliferation and angiogenesis, and inducing extracellular matrix remodeling.

Acupuncture benefits DF not only for its therapeutic effects but also demonstrates some preventive effects. It is important because reducing the incidence will significantly reduce the cost of DF treatment.

MOXIBUSTION

The mechanism of moxibustion-based therapy is similar to that of acupuncture, and it has complementary therapeutic effects to acupuncture. It has been verified that the smoke and heat of moxibustion have a role in promoting wound healing *via* inhibiting the inflammatory response and promoting the formation of collagen fibers, granulation tissues, and capillaries[56]. The expression of TGF-β in wound tissue is significantly increased after moxibustion intervention, indicating the promotion of fibroblast proliferation and rapid formation of granulation in the early stage[57]. After six moxibustion interventions in a rat model, Kan *et al*[58] found that fibroblasts and collagen fibers in the wound tissue were more closely arranged, and neovascularization was richer. They demonstrated that moxibustion ended the inflammatory stage by regulating proinflammatory cytokines and initiated the repair stage in advance. Moreover, the content of VEGF and VEGF in the serum of rats after the intervention was significantly increased[59]. However, some studies have raised doubts. Alonso *et al*[60] observed that acupuncture and moxibustion downregulated TGF-β1 and VEGF in adult female Wistar rats, but they still believed that moxibustion and acupuncture could stimulate fibroblast proliferation and neovascularization. Although many scholars have reported that moxibustion can promote wound healing, there is still no unified statement on its specific mechanism. Some scholars have even come to the opposite conclusion. Therefore, more basic and clinical research is needed to unveil the specific mechanism and justify the efficacy of moxibustion.

MASSAGE

Before the appearance of TCM decoction, ancient Chinese people started to use massage for disease prevention and treatment. Since massage needs to be administered at specific locations, when there are ulcers, massage will increase the pain and the risk of infection, so massage is mainly used for adjuvant treatment of diabetic peripheral neuropathy (DPN) and early DF. DPN is a significant risk factor for DF [61]. Nerve conduction studies are considered the gold standard in clinical research for DPN. Nerve conduction velocity (NCV) detects peripheral nerve conduction dysfunction caused by segmental demyelination and axonal damage and is usually slowed in DPN[62]. A recent meta-analysis of 3284 patients showed that TCM bath combined with acupoint massage improved the sensory and motor NCV and decreased neurological syndrome score in DPN[63].

Massage even improved the general condition of DM patients. Zarvasi *et al*[64] found that blood glucose significantly decreased and insulin levels increased after the self-acupressure intervention. After three years of acupressure treatment, the levels of total cholesterol, triglyceride, and low-density lipoprotein-cholesterol significantly decreased, and the level of high-density lipoprotein-cholesterol increased[65]. Massage appears to be beneficial not only for DF but also for the control of hyperlipidemia. It should be noted that since it is challenging to perform massage in animals, the specific mechanisms are hard to reveal.

ACUPOINT INJECTION

Acupoint injection is a common treatment method in TCM. Either injection of Chinese herbal extracts (*e.g.*, Danshen injection and Fufang Danggui injection) or conventional medicines (*e.g.*, mecobalamin, vitamin B1, and anisodamine) at specific acupoints are available[66]. Applying electroacupuncture after methylcobalamin injection at Sanyinjiao (SP6) can restore ulnar and tibial nerve motor NCV and sensory NCV in patients with DPN[67]. A systematic review of 1071 Chinese DPN patients showed that acupoint injection of Chinese herbal extracts at Zusanli (ST36) was safe and may reduce pain and improve nerve afferent velocity compared with intramuscular injection of the same drug[66]. However, the trials included in this review were of low quality. Therefore, higher quality clinical trials are necessary to delineate the safety and efficacy of acupoint injection as adjuvant therapy for DF.

FOOT BATH

TCM foot bath using decoctions increases blood circulation for Grade 0 DF[68]. Additionally, herbal foot baths can improve local microcirculation and regulate skin permeability to increase drug absorption, thus effectively increasing drug concentration[69,70]. Recently, clinical trials have been designed to

examine the efficacy and safety of TCM foot baths[68,71]. Nevertheless, foot baths can spread infection at the ulcer site in patients with chronic limb ulcers and increase the rate of toe loss (53%) and major amputation (30%)[72]. Consequently, the choice of foot bath treatment for DF needs to be carefully considered by clinicians due to its double-sided effect.

FUMIGATION

Chinese herbal fumigation is a kind of external treatment of TCM, which can relax muscles and tendons and remove obstructions from meridians, activating blood to eliminate stagnation[73]. Cuyuxunxi prescription is a Chinese herbal fumigant widely applied to wash surgical wounds after anal fistulotomy, potentially promoting wound healing and antagonizing infection[74]. Zhuyuan decoction fumigation is an effective treatment to relieve the symptoms of patients with chronic sinusitis[75]. In addition, fumigation reduces knee osteoarthritis swelling and pain by inhibiting the expression of pro-inflammatory factors, promoting blood reflux, and reducing skin sensory nerve excitability[76]. Meanwhile, fumigation smoke and heat can promote wound healing in rats by inhibiting inflammatory responses and ameliorating the formation of collagen fibers, granulation tissue, and capillary status[56]. An ongoing systematic review will evaluate the effectiveness and safety of TCM fumigation in DPN [77]. Fumigation may be an effective therapeutic measure for DFU due to its anti-infective, inflammation-inhibiting, and wound-healing effects.

PROSPECTIVE

DF can be divided into neurologic, ischemic, or neuroischemic according to the International Working Group on the Diabetic Foot (IWGDF)[78] while The Society for Vascular Surgery Lower Extremity Threatened Limb (SVS WIfI) classification system classifies DF into four grades: Grade 0, 1, 2, and 3 (Table 1)[79]. For grade 2 or 3 DF, amputation and hemodynamic reconstruction are often required. Therefore, TCM adjuvant therapy is mainly used for grades 0 and 1 DF. As shown in Figure 1, for grade 0 or 1 DF caused by neuropathy (usually DPN), foot bath, acupoint injection, and massage are optional treatment modalities because they can accelerate the sensory and motor NCV in the lower limbs. External application, moxibustion, fumigation, acupuncture, massage, and foot bath can increase blood flow in the lower extremities and promote neovascularization in the local wound for ischemic grade 0-1 DF. For neuroischemic grade 0 or 1 DF, massage, footbath, or a combination of other adjunctive therapies, can be chosen. For all DF (including grades 2 and 3), massage is an optional adjunctive therapy that regulates local and systemic metabolism (including blood glucose and lipids). In addition, topical application, moxibustion, and acupuncture can promote wound healing in grade 1 DF, and fumigation may be an effective anti-infection modality when local infection occurs. In conclusion, selecting appropriate TCM adjunctive therapy for early DF(grades 0 and 1) will positively affect patients, but it should be noted that foot baths may lead to skin maceration and increase the rate of amputation.

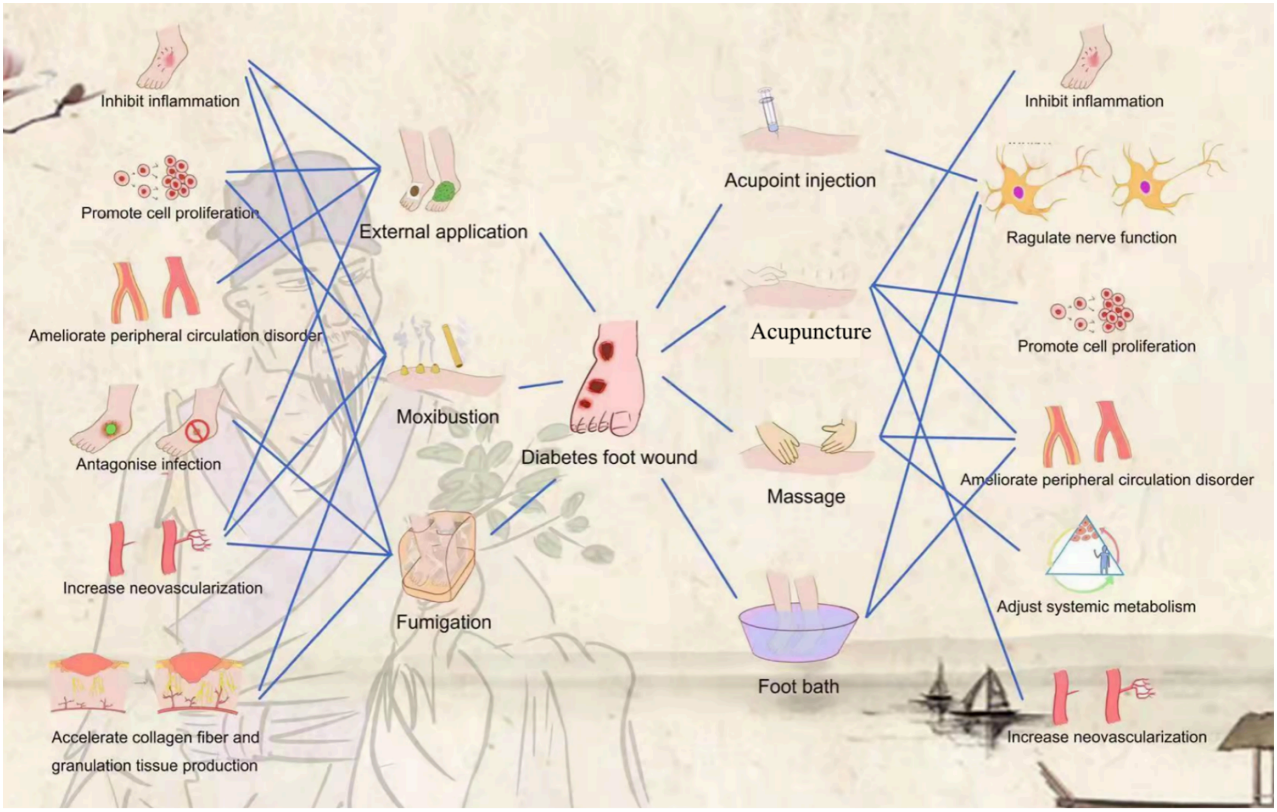
Chinese herbal medicine treatment is distinguished by its multi-target and multi-level nature. With the application of network pharmacology to the study of TCM herbal formulations in recent years, the active constituents of herbal medications and their unique targets of action have been discovered, providing a theoretical foundation for their clinical use[80]. Future studies may focus on new wound dressings utilizing medicinal plant extracts or their purified active components[81]. Nonetheless, we must not overlook the fact that the precise mechanism of action of TCM requires additional investigation. In addition, there is no research on the effectiveness of TCM in preventing DF. “Treating the untreated” has been a critical area of concern for TCM, and scientific randomized controlled trials (RCTs) can be used to confirm its risk-benefit ratio in the prevention of DF is also necessary.

CONCLUSION

DF is a common complication of diabetes. There are many adjunctive therapies in TCM that can be applied to DF. Some have been proven effective, while others require more research. Animal experiments have confirmed that TCM adjuvant therapy can promote DFU wounding healing by inhibiting nonphysiological inflammation *via* down-regulating AGEs, RAGE, TNF-α, and IL-1β, promoting neovascularization *via* up-regulating VEGF and PDGF, inducing extracellular matrix remodeling, improving local blood circulation, and accelerating the production of collagen fibers and granulation tissue. In the future, more high-quality research is needed to demonstrate and popularize the application of TCM adjuvant therapy in DF.

Table 1 Society for Vascular Surgery Lower Extremity Threatened Limb (SVS WIfI) classification system

Grade	Ulcer	Gangrene
0	No ulcer	No gangrene
1	Small, shallow ulcer(s) on distal leg or foot; no exposed bone, unless limited to distal phalanx	No gangrene
2	Deeper ulcer with exposed bone, joint or tendon; generally not involving the heel; shallow heel ulcer, without calcaneal involvement	Gangrenous changes limited to digits
3	Extensive, deep ulcer involving forefoot and/or midfoot; deep, full thickness heel ulcer ± calcaneal involvement	Extensive gangrene involving forefoot and/or midfoot; full thickness heel necrosis ± calcaneal involvement



DOI: 10.4239/wjd.v13.i10.851

Figure 1 Categories of traditional Chinese medicine as adjuvant therapy for diabetic foot wound and related mechanisms.

ACKNOWLEDGEMENTS

We thank the Fudan University Committee of the Communist Youth League for the guidance of diagram.

FOOTNOTES

Author contributions: Liu FS wrote the paper; Li Y, Guo XS, and Liu RC performed the collected the data; Li Z reviewed the article; all authors have read and approved the final version.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YX

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 861-876

DOI: 10.4239/wjd.v13.i10.861

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Correlation between gut microbiota and glucagon-like peptide-1 in patients with gestational diabetes mellitus

Yun-Yi Liang, Ling-Yu Liu, Yan Jia, Yi Li, Jie-Na Cai, Yi Shu, Jing-Yi Tan, Pei-Yi Chen, Hong-Wei Li, Hui-Hua Cai, Xiang-Sheng Cai

Specialty type: Microbiology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Houri H, Iran; Janvilisri T, Thailand

Received: March 26, 2022
Peer-review started: March 26, 2022
First decision: June 11, 2022
Revised: June 25, 2022
Accepted: August 24, 2022
Article in press: August 24, 2022
Published online: October 15, 2022

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) places both the mother and offspring at high risk of complications. Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of GDM. However, it is still unclear whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients.

AIM

To explore the correlation between the gut microbiota and blood biochemical traits, particularly GLP-1, in GDM patients.

METHODS

The V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was sequenced

based on the fecal samples of 35 pregnant women with GDM and was compared to that of 25 pregnant women with normal glucose tolerance (NGT).

RESULTS

The results showed that *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Spearman's correlation analysis was performed to identify the relationships between microbiota genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were significantly negatively correlated with glucose. *Ruminococcaceae_UCG-002* was significantly negatively correlated with hemoglobin A1c. *Bacteroides* was significantly positively correlated with glucose. *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were significantly positively correlated with GLP-1. A random forest model showed that 20 specific genera plus glucose provided the best discriminatory power, as indicated by the area under the receiver operating characteristic curve (0.94).

CONCLUSION

The results of this study reveal novel relationships between the gut microbiome, blood biochemical traits, particularly GLP-1, and GDM status. These findings suggest that some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM.

Key Words: Gut microbiome; Glucagon-like peptide-1; Gestational diabetes mellitus; Glucose

Core Tip: Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of gestational diabetes mellitus (GDM). However, it is still unclear whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients. To the best of our knowledge, this is the first study to analyze the relationship between GLP-1 and the gut microbiota in patients with GDM, and this is the first report on the relationship between *Paraprevotella*, *Roseburia*, and *Faecalibacterium* and glucose in GDM, and the first report on the associations between GLP-1 and genera including *Sutterella, Oscillibacter*, and *Bifidobacterium* in GDM.

Citation: Liang YY, Liu LY, Jia Y, Li Y, Cai JN, Shu Y, Tan JY, Chen PY, Li HW, Cai HH, Cai XS. Correlation between gut microbiota and glucagon-like peptide-1 in patients with gestational diabetes mellitus. *World J Diabetes* 2022; 13(10): 861-876
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/861.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.861

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as abnormal glucose tolerance in pregnancy[1]. It is one of the most common complications of pregnancy. The incidence of GDM has increased due to lifestyle changes, increasing maternal age, and changes to the GDM diagnostic criteria. The incidence of GDM is reported to be 13.20%. GDM is closely related to the occurrence of perinatal maternal and neonatal complications, and also significantly increases the risk of long-term metabolic diseases in pregnant women and newborns. The pathogenesis of GDM is complex and has not yet been comprehensively elucidated. Timely diagnosis and intervention are of great significance to the long-term health of pregnant women and their fetuses.

In recent years, with increased research and understanding of gastrointestinal hormones, their roles in the occurrence and development of GDM have attracted growing attention. Research suggests that GDM patients exhibit insufficient glucagon-like peptide-1 (GLP-1) secretion during pregnancy and after delivery relative to their blood glucose level. Bonde *et al*[2] found that the postprandial GLP-1 level of pregnant women was decreased, especially in GDM patients. However, with the recovery of blood glucose homeostasis after delivery, postprandial GLP-1 secretion gradually returned to normal. Kosinski *et al*[3] confirmed that decreased GLP-1 in patients with GDM is reversible. Changes in GLP-1 levels may be related to insulin resistance (IR) as a result of high blood glucose levels. However, it

cannot be ruled out that changes in GLP-1 levels may be involved in the occurrence and development of GDM.

Evidence indicates that the gut microbiota is closely related to GDM[4]. The mechanism underlying the effect of probiotics in diabetes has not yet been fully elucidated, but it may be related to reductions in oxidative stress, regulation of the immune response, reductions in inflammation, and regulation of the gut microbiota[5,6]. In addition, probiotics can also reduce postprandial blood lipid levels and improve the absorption of antioxidants, which are related to oxidative stress[7]. Numerous studies have demonstrated that GLP-1 has insulin tropic and antioxidant effects[8-10]. Since GLP-1 and the gut microbiota each play roles in GDM, is there a correlation between the two?

Both clinical and animal studies have reported correlations between changes in the GLP-1 level and changes in the gut microbiota after gastrointestinal bypass surgery in type 2 diabetes mellitus (T2DM) patients or mice[11]. Therefore, it is speculated that GLP-1 may regulate blood glucose by regulating the number and structure of gut microbiota. Several authors have argued that GLP-1 may play a role in regulating blood glucose by increasing the diversity of the gut microbiota[12] and increasing the proportion of probiotics. Together, the current literature provides a comprehensive explanation of the hypoglycemic mechanism of GLP-1 and a reliable experimental basis for the study of GDM therapeutic targets and therapeutic drugs based on GLP-1. Accordingly, one study found that bifidobacteria improved insulin sensitivity by increasing the production of GLP-1[13].

The rapid increase in the prevalence of GDM in recent years cannot be easily explained by genetic factors; thus, it has been hypothesized that environmental factors may play a more important role. The gut microbiota constitutes an important environmental factor. GLP-1, as the most important representative of gastrointestinal hormones, may also be involved in the pathogenesis of GDM. The gastrointestinal microbiota and gastrointestinal hormones share the same root, are inseparable, influence and restrict each other, and jointly participate in the occurrence, development, and prognosis of GDM. Thus, a comprehensive study of the correlations between changes in the gut microbiota and GLP-1 will help to further clarify the pathogenesis of GDM. This is of great significance for the prevention, treatment, and prognosis of GDM, and may provide a novel and sensitive index for the clinical evaluation of GDM.

MATERIALS AND METHODS

Subjects

Patients were screened for GDM in the obstetric outpatient department according to the GDM diagnostic criteria (2014). Thirty-five patients with GDM were randomly selected from the patients who met the diagnostic criteria for GDM; these patients formed the GDM group. A further 25 pregnant women with normal glucose tolerance (NGT) were selected as the NGT group. Each subject provided written informed consent before inclusion in the study. The study was approved and carried out in accordance with the guidelines of the Ethics Committee of Nanhai District People's Hospital of Foshan.

The inclusion criteria were as follows: 18-45 years old, being female, and any education level. Based on the diagnostic criteria for gestational diabetes (2014), during the 24-28th week of gestation, the 75 g oral glucose tolerance test (OGTT) was used to measure each patient's blood glucose levels before, 1 h after, and 2 h after consuming sugar. If the patient's blood glucose level reached or exceeded 5.1, 10.0, or 8.5 mmol/L, respectively, they were deemed to have GDM and were eligible for inclusion in the GDM group.

The exclusion criteria were as follows: (1) History of chronic digestive system disorder; (2) history of treatment with GLP-1 analogues or GLP-1 receptor agonists; (3) history of cardiac, renal, or liver dysfunction; (4) multiple pregnancy; (5) pregnancy-induced hypertension syndrome, placental insufficiency, placenta previa, placental abruption, pelvic or soft birth canal abnormalities, or other pregnancy complications; (6) history of mental disorders; (7) exposure to a large amount of radiation, chemical poisons, or drugs that can affect the fetus during pregnancy; (8) tumor history or history of radiotherapy and chemotherapy within the past 6 mo; (9) participation in other research studies; (10) patients lost to follow-up due to various reasons, including the occurrence of other serious diseases during the study; and (11) consumption of antibiotics or probiotics within 1 mo prior to admission.

Sample collection and testing

Fresh fecal samples were collected from the participants and immediately frozen in a refrigerator at -80 °C. After collection of all samples, they were sent to the Treat Gut company for 16S rDNA sequencing.

Blood samples were collected after fasting and then 1 h and 2 h following consumption of sugar. Plasma glucose (Glu), glycosylated serum protein (GSP), low-density lipoprotein (LDL), uric acid (UA), hemoglobin (HB), total cholesterol (TCH), triglyceride (TG), and high-density lipoprotein (HDL) were determined with a Beckman AU5800 fully automatic biochemical analyzer. Glycosylated hemoglobin A1c (HbA1c) was determined using an ADAMS™ A1c HA-8180 automatic glycosylated hemoglobin analyzer. Insulin (INS), thyroid-stimulating hormone (TSH), and free tetraiodothyronine (FT4) were measured with a Maglumi2000plus automatic chemiluminescence immunoanalyzer.

The active forms of GLP-1 in the plasma samples of patients with and without GDM were measured using a GLP-1 (active) ELISA kit (ELabscience, Wuhan, Hubei Province, China).

Bacterial 16S rRNA gene sequencing

The total genomic DNA of each sample was extracted using a fecal genomic DNA extraction kit (Tiangen Company). Sixteen S rDNA sequencing was performed by PCR amplification of V4 variable regions (39 to 297 base pairs), and a purified product library was established. The library construction steps followed the library construction method of the Illumina sequencing platform. The sequencing analysis was as follows. First, the Illumina Miseq 2 × 300bp paired-end sequencing data were analyzed. According to the barcode information, the samples were distinguished. Then, the data were merged, spliced, and filtered, and quality control analysis was conducted, including Q20 and Q30 scores. The final clean data were analyzed by operational taxonomic unit (OTU) cluster analysis and species taxonomy.

Microbiome data

The data were filtered using Mothur software and clustered into OTUs (species) at a similarity level of 97% using Quantitative Insights into Microbial Ecology (QIIME) software version 1.80[14]. Based on the OTU analysis, the Ace, Shannon, observed species, Simpson, Chao1, and J indices were calculated as alpha diversity metrics. To compare the microbial composition between the samples, beta diversity analysis was performed using principal component analysis (PCA) and principal coordinate analysis (PCoA). Analysis of similarities (ANOSIM) was applied to evaluate the statistical significance of differences between the groups. A linear discriminant analysis (LDA) effect size (LEfSe) method was employed to evaluate any differences in the gut microbe between the groups.

Statistical analysis

GraphPad Prism (version 7.0) and R version 3.0.2 (R Foundation for Statistical Computing) were using for statistical analyses. The measurement data are expressed as the mean ± SD. Differences between groups were analyzed using oneway ANOVA. The differences were considered statistically significant at $P < 0.05$. Random-forest classification was performed for discriminating the samples from different groups using the R package "random forest". The model was employed for five-fold cross-validation of the relative species abundance profile. Case probabilities were calculated by drawing receiver operating characteristic (ROC) curves.

RESULTS

Characteristics of the study population

GDM was diagnosed in 35 women based on fasting or oral glucose-stimulated hyperglycemia, or a combination of the two. Markers of glucose and insulin homeostasis were higher in the GDM group compared with the NGT group (Table 1). Individuals with GDM also had higher hemoglobin A1C ($P = 0.003$) and fasting blood glucose levels ($P < 0.001$). There were no significant differences in pre-pregnancy body weight, BMI, UA, TCH, TG, HDL, LDL, TSH, or FT4 between the two groups.

OTU distributions

In this study, the OTUs annotated included 14 phyla, 62 families, and 214 genera of gut microbiota; the similarity among samples was 97% (Figure 1A). The total number of OTUs of the NGT group (at the 97% similarity level) was 652, and for the GDM group, it was 619. Venn diagram shows that 560 OTUs were shared by the NGT and GDM groups (Figure 1B).

Alpha and beta diversities

The observed species index of the GDM group was significantly difference from that of the NGT group (25; $P = 0.044$). The Chao1 richness index of the GDM group was significantly different from that of the NGT group (43; $P = 0.004$). The ACE index of the GDM group differed significantly from that of the NGT group (25; $P = 0.0055$) (Figure 2). There were no significant differences in the Shannon, Simpson, or J indices between the GDM group and NGT group (Shannon, $P = 0.65$; Simpson, $P = 0.9$; J, $P = 0.91$; Figure 2A). PCA and PCoA indicated that the gut microbiota in GDM patients differed significantly from that of the NGT subjects. There was no difference in the gut microbiota structure between the groups (ANOSIM, $r = 0.019$, $P = 0.2232$). NMDS cluster analysis indicated marked differences between the GDM patients and NGT subjects (Figure 2B).

Taxonomy

The composition of gut microbiota was different between the groups at the phylum, family, and genus levels. At the phylum level, *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and *Tenericutes* were common phyla in both the GDM group and NGT group,

Table 1 Clinical variables of gestational diabetes mellitus patients and healthy controls

Variable	Control (25)	GDM (31)	*P* value
Age (yr)	28.42 (3.11)	30.18 (3.26)	0.055
Pre-body weight (kg)	52.42 (7.68)	55.18 (6.48)	0.165
Height (cm)	160.13 (5.44)	157.88 (4.10)	0.063
Pre-BMI	20.73 (2.85)	22.2 (2.37)	0.054
GLU (mmol/L)	4.49 (0.38)	5.77 (0.95)	3.53×10^{-7}
GLP-1 0 h (ug/L)	67.72 (22.89)	75.45(23.23)	0.223
GLP-1 1 h (ug/L)	75.33 (26.14)	84.34 (19.84)	0.099
GLP-1 2 h (ug/L)	71.75 (23.83)	79.21 (24.20)	0.312
OGTT 0 h (mmol/L)	4.47 (0.39)	5.74 (0.99)	3.53×10^{-7}
OGTT 1 h (mmol/L)	7.77 (1.55)	11.23 (2.95)	3.00×10^{-6}
OGTT 2 h (mmol/L)	6.26 (0.87)	9.64 (3.12)	3.96×10^{-6}
Insulin 0 h (uIU/mL)	9.78 (3.41)	14.03 (15.93)	0.239
Insulin 1 h (uIU/mL)	85.85 (43.99)	64.52 (39.67)	0.061
Insulin 2 h (uIU/mL)	57.51 (39.36)	66.52 (45.21)	0.675
GSP (mmol/L)	1.68 (0.35)	1.97 (0.62)	0.054
HbA1c (%)	5.04 (0.30)	5.79 (1.12)	0.003
UA (umol/L)	279.52(68.47)	263.80(81.27)	0.463
TCH (mmol/L)	5.40 (1.06)	5.35 (1.08)	0.993
TG (mmol/L)	1.82 (0.72)	2.45 (1.54)	0.051
HDL (mmol/L)	2.11 (0.52)	2.08 (0.63)	0.946
LDL (mmol/L)	2.41 (0.89)	2.34 (0.82)	0.739
TSH (uIU/mL)	1.82 (3.43)	1.22 (1.24)	0.337
FT4 (pg/mL)	11.39 (3.27)	11.77 (1.96)	0.598

OGTT: Oral glucose tolerance test; GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; Glu: Glucose; GSP: Glycosylated serum protein; HbA1c: Hemoglobin A1c; UA: Uric acid; TCH: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TSH: Thyroid-stimulating hormone; FT4: Free tetraiodothyronine.

accounting for 98.81% and 98.58% of the gut bacteria of each group, respectively (Figure 3). The GDM group had a lower abundance of *Firmicutes* (31.4% *vs* 33.2%), *Verrucomicrobia* (0.18% *vs* 1.01%, $P < 0.05$), *Synergistetes* (0.003% *vs* 0.110%, $P < 0.01$), and *Tenericutes* (0.05% *vs* 0.08%), and a higher abundance of *Bacteroidetes* (63.50% *vs* 60.81%), *Proteobacteria* (3.03% *vs* 2.56%), and *Fusobacteria* (0.33% *vs* 0.29%), compared to the NGT group. The ratio of *Firmicutes* to *Bacteroidetes* was decreased in the GDM group compared to the NGT group (0.49 *vs* 0.54).

At the family level, a greater number of different families were identified between the two groups (Figure 4). Fifty-five and 54 of the dominant families were detected in the GDM group and NGT group, respectively. *Bacteroidaceae* (phylum *Bacteroidetes*), *Prevotellaceae* (phylum *Bacteroidetes*), *Acidaminococcaceae* (phylum *Firmicutes*), *Veillonellaceae* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Ruminococcaceae* (phylum *Firmicutes*), *Enterobacteriaceae* (phylum *Proteobacteria*), and *Tannerellaceae* (phylum *Bacteroidetes*) had the highest relative abundance in the GDM group, while *Bacteroidaceae*, *Prevotellaceae*, *Acidaminococcaceae*, *Veillonellaceae*, *Lachnospiraceae*, *Enterobacteriaceae*, *Ruminococcaceae*, and *Rikenellaceae* were the eight most abundant families in the NGT group.

The bacterial taxa whose levels differed significantly between the two groups were identified by LEfSE analysis (Figure 4). At the family level, *Atopobiaceae*, *Eggerthellaceae*, *Streptococcaceae*, *Christensenellaceae*, *Clostridiaceae*, *Bifidobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were significantly more abundant in the NGT group than in the GDM group.

At the genus level, bacterial genera exhibited significant differences between the two groups (Figure 5). In the NGT group, *Bacteroides* (phylum *Bacteroidetes*), *Prevotella_9* (phylum *Bacteroidetes*), *Phascolarctobacterium* (phylum *Firmicutes*), *Megasphaera* (phylum *Firmicutes*), *Megamonas* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Escherichia-Shigella* (phylum *Proteobacteria*), and

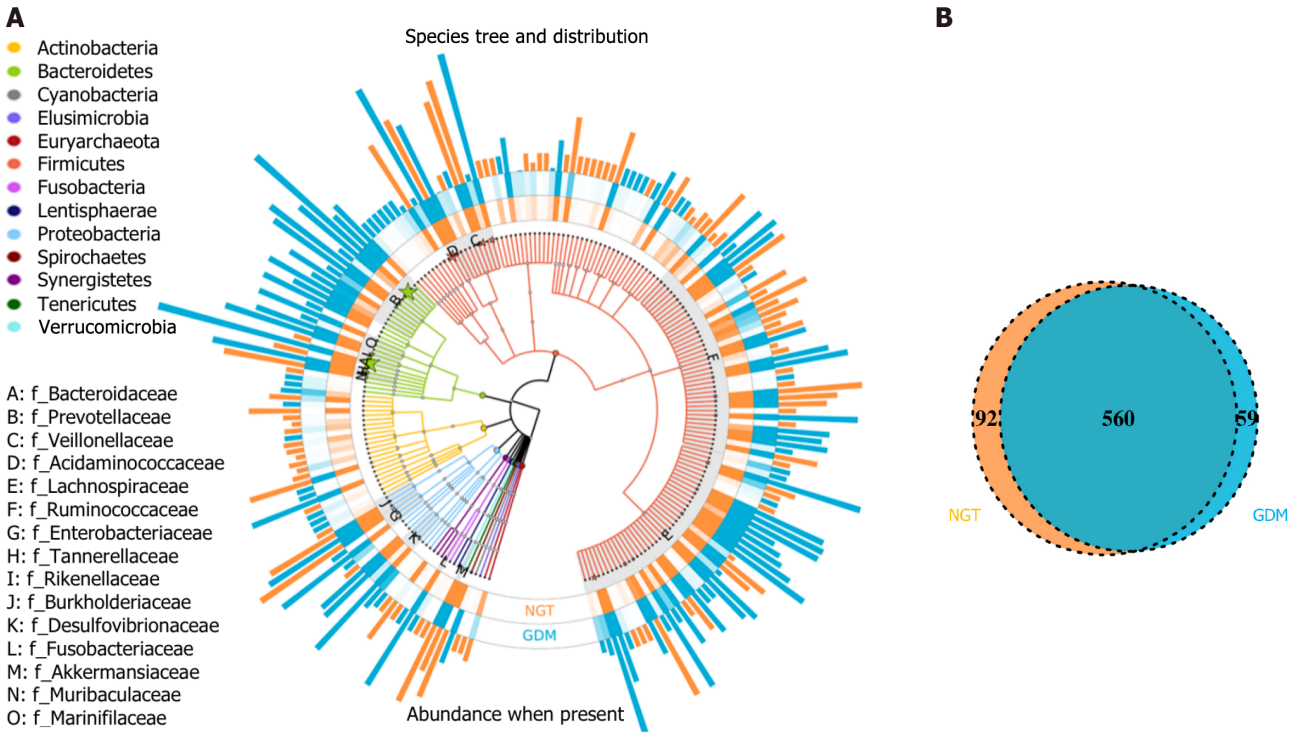

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Figure 1 Operational taxonomic units distributions. A: Species tree and distribution of the gut microbial community; B: Venn diagram showing the common or specific operational taxonomic units between the groups. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

Prevotella_2 (phylum *Bacteroidetes*) were the eight most dominant genera. The eight most dominant genera in the GDM group were *Bacteroides* (phylum *Bacteroidetes*), *Prevotella_9* (phylum *Bacteroidetes*), *Megamonas* (phylum *Firmicutes*), *Phascolarctobacterium* (phylum *Firmicutes*), *Lachnospiraceae* (phylum *Firmicutes*), *Megasphaera* (phylum *Firmicutes*), *Prevotella_2* (phylum *Bacteroidetes*), and *Parabacteroides* (phylum *Bacteroidetes*).

Ruminococcaceae_UCG-002, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group ($P < 0.05$). *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group ($P < 0.05$). *Prevotella_9*, *Oscillibacter*, *Roseburia*, and *Faecalibacterium* were slightly more abundant in the NGT group than in the GDM group.

Functional profiling of the gut microbiome

The cluster of orthologous groups (COG) categories and KEGG pathways were compared between the GDM and NGT groups. Figure 6A shows that three functional KEGG pathways differed between the GDM group and NGT group, including the glycosphingolipid biosynthesis-globo series, synthesis and degradation of ketone bodies, and renal cell carcinoma pathways. Figure 6B shows that 20 COG categories differed between the GDM group and NGT group, including the phosphotransferase system, galactitol-specific IIC component, metal-dependent proteases with possible chaperone activity, uncharacterized protein, homolog of phage Mu protein gp30, uncharacterized protein conserved in bacteria, Acyl-CoA dehydrogenases, putative virion core protein (lumpy skin disease virus), predicted phosphohydrolase, large-conductance mechanosensitive channel, uncharacterized conserved protein, uncharacterized protein predicted to be involved in DNA repair, predicted permease, DMT superfamily, nicotinic acid mononucleotide adenylyltransferase, amidases related to nicotinamidase, histone acetyltransferase, plasmid maintenance system antidote protein, uncharacterized conserved protein, DNA polymerase III, alpha subunit, uncharacterized protein conserved in bacteria, antirestriction protein, NA polymerase III, and alpha subunit (Gram-positive type).

Correlations between blood biochemical traits and gut composition

Spearman's correlation analysis was performed to identify whether the different dominant genera were associated with blood biochemical traits in the second trimester of pregnancy (Figure 7). *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were negatively correlated with Glu ($P < 0.05$). *Ruminococcaceae_UCG-002* was negatively correlated with HbA1c ($P < 0.05$). *Clostridium_sensu_stricto_1*, *Desulfovibrio*, and (*Ruminococcus*)*_torques_group* were negatively correlated with pre-pregnancy body weight ($P < 0.05$). *Phascolarctobacterium* was negatively correlated with HDL ($P < 0.05$). *Ruminococ-*

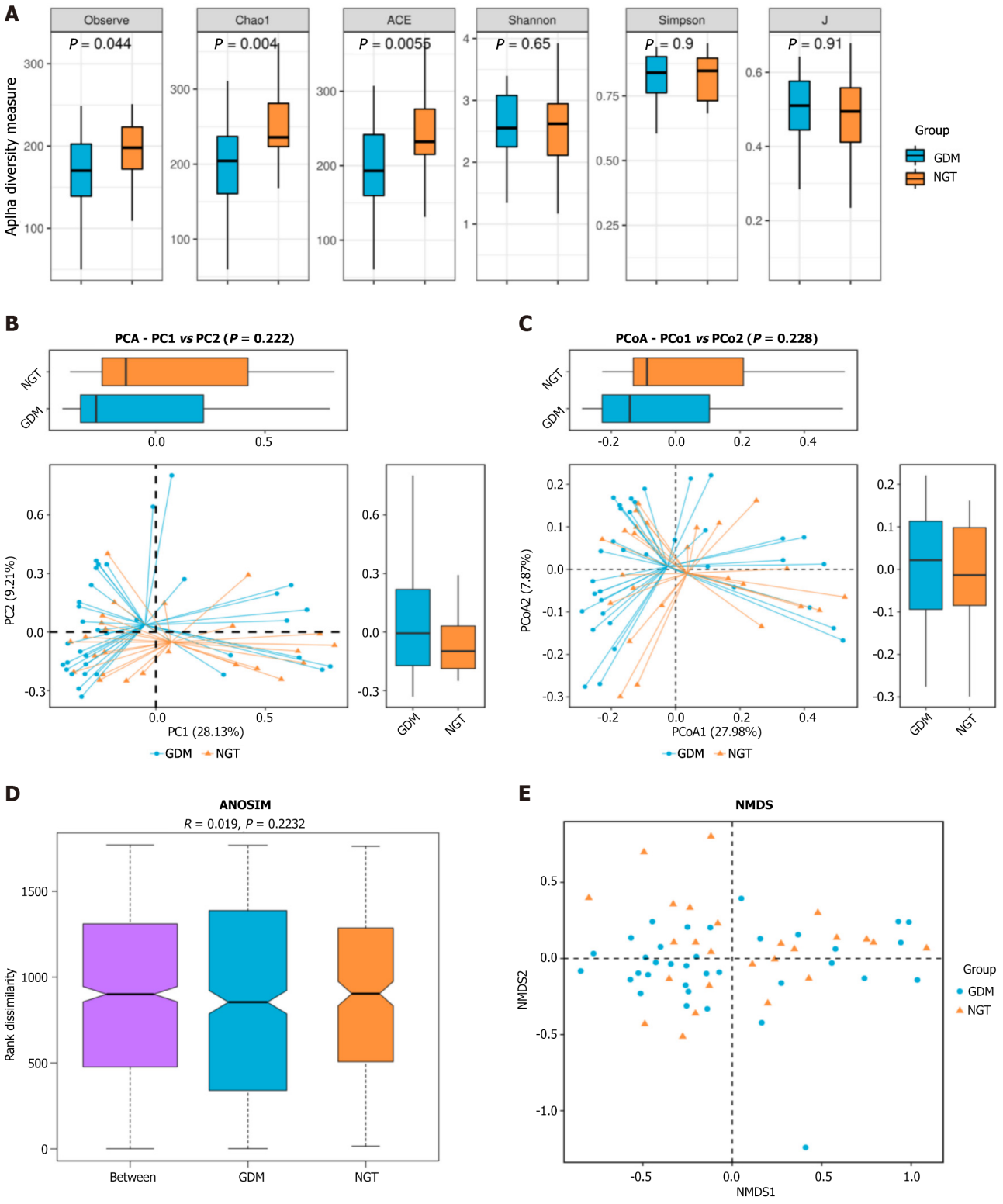

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Figure 2 Gut microbiota alpha and beta diversity indices in patients with gestational diabetes mellitus. A: Gut microbiota alpha diversity indices in patients with gestational diabetes mellitus. The Observed_species, ACE, Chao1, Simpson, Shannon, and J values are shown; B: Principal component analysis score plot based on the relative abundance of operational taxonomic units (97% similarity levels); C: Principal coordinate analysis; D: Similarities analysis; E: Non-metric multidimensional scaling. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; PCoA: Principal coordinate analysis; PCA: Principal component analysis.

caceae_UCG-003 and *Faecalibacterium* were negatively correlated with height ($P < 0.05$). *Lachnoclostridium* and *Lachnospiraceae_NK4A136_group* were positively correlated with age ($P < 0.05$). *Bacteroides* was significantly positively correlated with Glu ($P < 0.01$). *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were positively correlated with GLP-1 ($P < 0.05$).

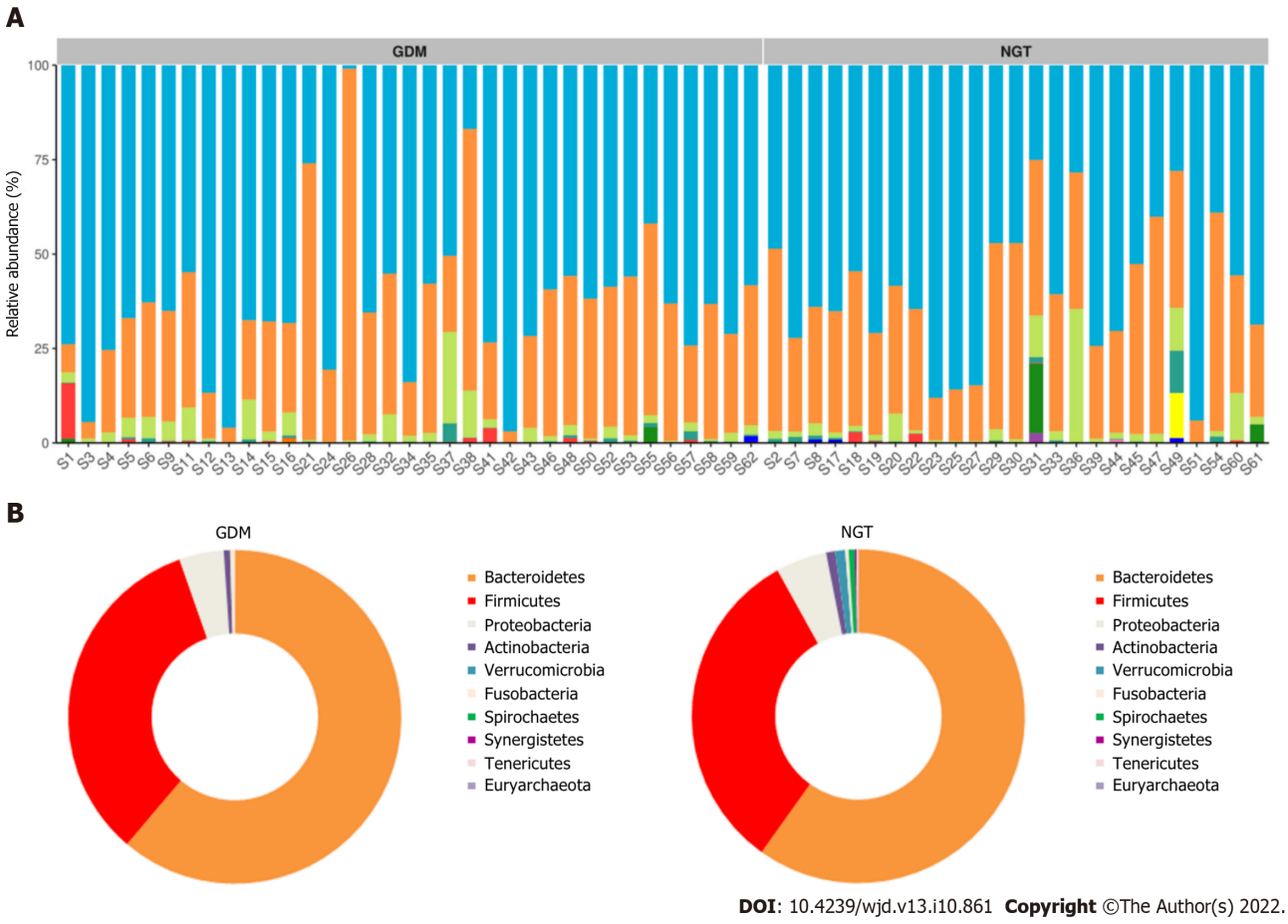

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Figure 3 Taxonomy. A: Top eight abundant species at the phylum level; B: Different bacteria were compared between each group at the phylum level. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

Roseburia was negatively correlated with OGTT (0 h), OGTT (1 h), and OGTT (2 h) ($P < 0.05$). *Faecalibacterium* was negatively correlated with OGTT (0 h) and OGTT (1 h) ($P < 0.05$). *Bacteroides* was positively correlated with OGTT (0 h), OGTT (1 h), OGTT (2 h), and GSP ($P < 0.05$). *Lachnoclostridium* was positively correlated with OGTT (1 h) and OGTT (2 h) ($P < 0.05$). *Sutterella* was positively correlated with GLP-1(0 h), GLP-1(1 h), GLP-1(2 h), and pre-pregnancy BMI ($P < 0.05$). *Oscillibacter* was positively correlated with GLP-1(0 h), GLP-1(1 h), and GLP-1(2 h) ($P < 0.05$). *Bifidobacterium* was positively correlated with GLP-1(0 h), GLP-1(1 h), OGTT (2 h), TG, and TCH ($P < 0.05$).

Gut microbiota–based prediction of GDM

Finally, random forest models were used to assess the ability of the genera abundance profiles to predict GDM status (Figure 8A). Twenty genera plus Glu provided the best discriminatory power, as indicated by the area under the receiver operating characteristic (AUROCC) value of 0.94. The value was higher than that achieved with a model including just 20 genera (the best AUC was 0.828) (Figure 8B). Further, models with 20 genera plus GLP-1, INS, or HbA1c had lower AUROCC values than the model with 20 genera plus Glu. The AUROCC values were 0.81, 0.8288, and 0.8502, respectively.

DISCUSSION

In recent years, the relationships between the gut microbiota and diabetes as well as other endocrine diseases have become research hotspots. Similarly, the characteristics of the gut microbiota among pregnant women with GDM have received widespread research attention. To date, research on GDM has focused on the correlation between the gut microbiota and blood glucose or insulin, but there is still a lack of research on the relationship between the gut microbiota and GLP-1. Many studies have reported that GLP-1 is closely related to the gut microbiota and short-chain fatty acids (SCFAs)[15-17], and changes in the gut microbiota directly affect the secretion of GLP-1, which, in turn, affects insulin and blood glucose. These are closely related to the occurrence of GDM. Therefore, we focused on the relationship between GLP-1 and the gut microbiota in GDM patients. To the best of our knowledge, it is the first report on the relationship between GLP-1 and the gut microbiota in patients with GDM.

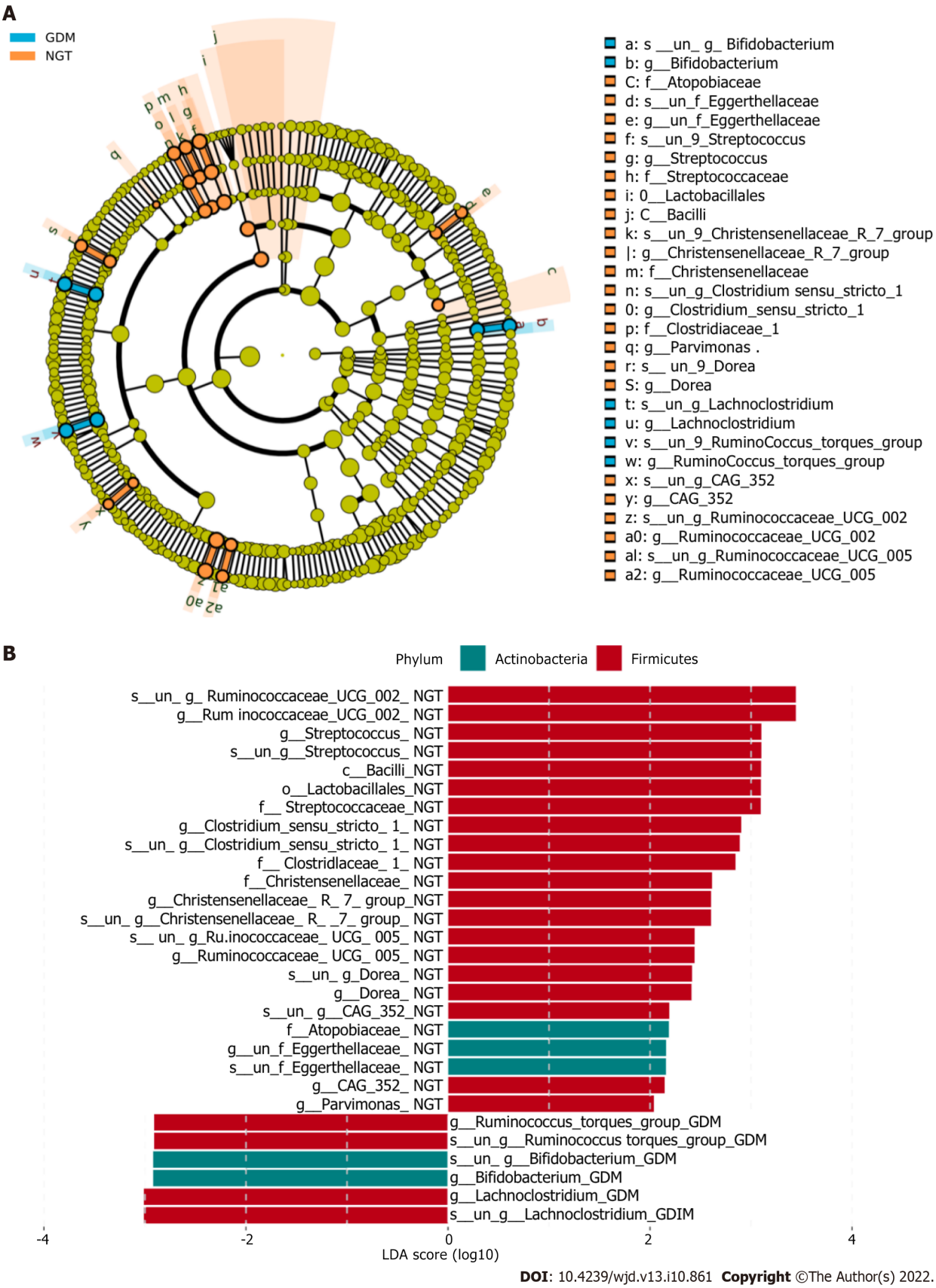

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Figure 4 LEfSE analysis to determine which bacterial taxa differ significantly between the groups. A: At the family level, a greater number of different families were identified between the normal glucose tolerance (NGT) and gestational diabetes mellitus groups (GDM); B: The bacterial taxa whose levels differed significantly between the NGT and GDM groups were identified by LEfSE analysis. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; LDA: Linear discriminant analysis.

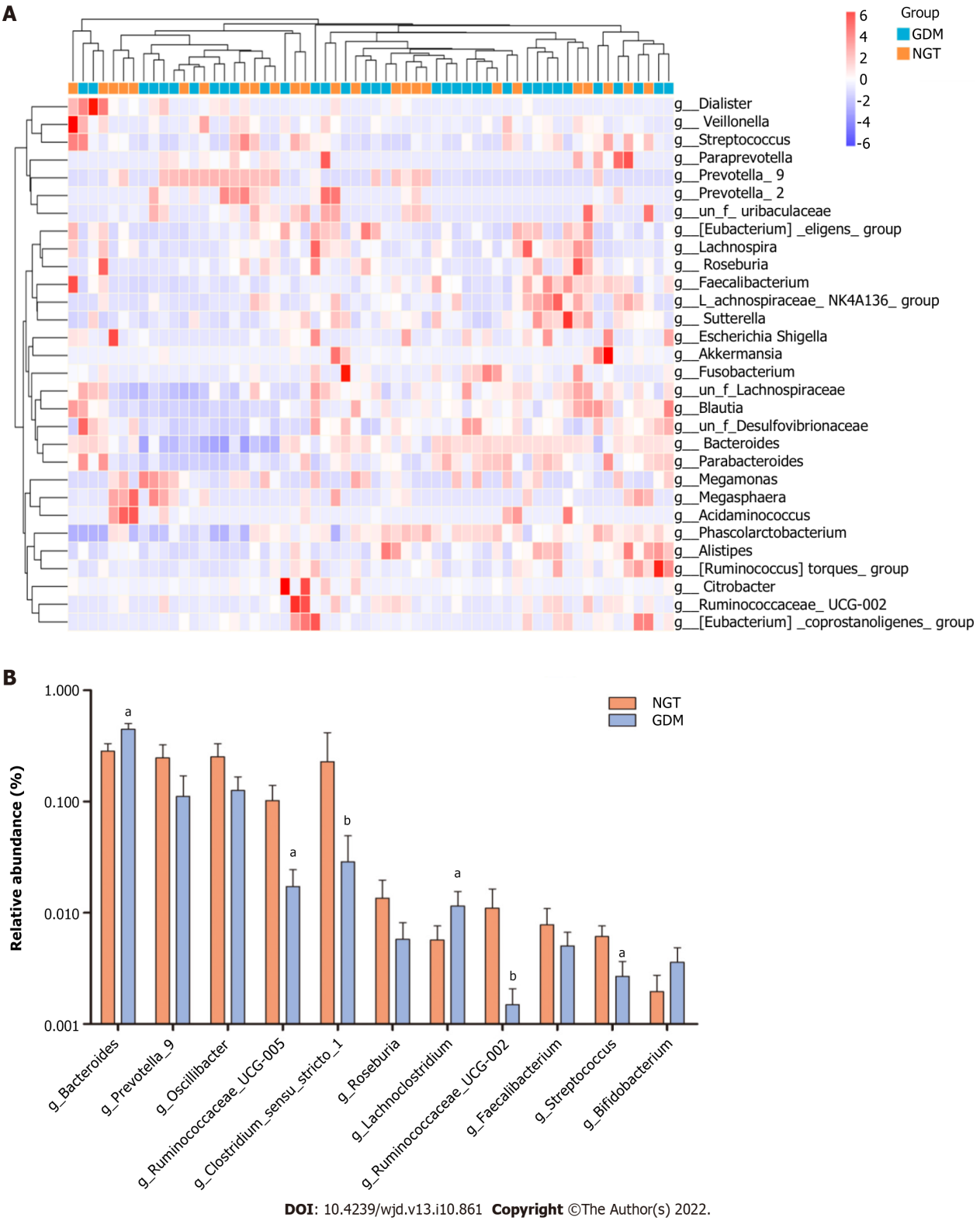

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Figure 5 Bacterial genera exhibit significant differences between the two groups. A: Heatmap showing the relative total abundance of the first 30 genera; B: Microbial community at the genus level between groups. $^{a}P < 0.05$, GDM group compared with NGT group. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

At the phylum level, the abundance of *Firmicutes* in the gut microbiota of the GDM group was lower than that in NGT group. *Firmicutes* are known to transform carbohydrates and undigested proteins into SCFAs, producing energy for the host organism. As a crucial SCFA, butyrate participates in the activation of multiple physiological signal pathways, including the proliferation and differentiation of regulatory T cells and anti-inflammatory activities[18,19]. Moreover, the GDM group exhibited reduced phylum levels of *Verrucomicrobia*, *Synergistetes*, and *Tenericutes* compared to the NGT group. Mucin-degrading bacteria *Verrucomicrobia* contribute to glucose homeostasis and intestinal health, and play a

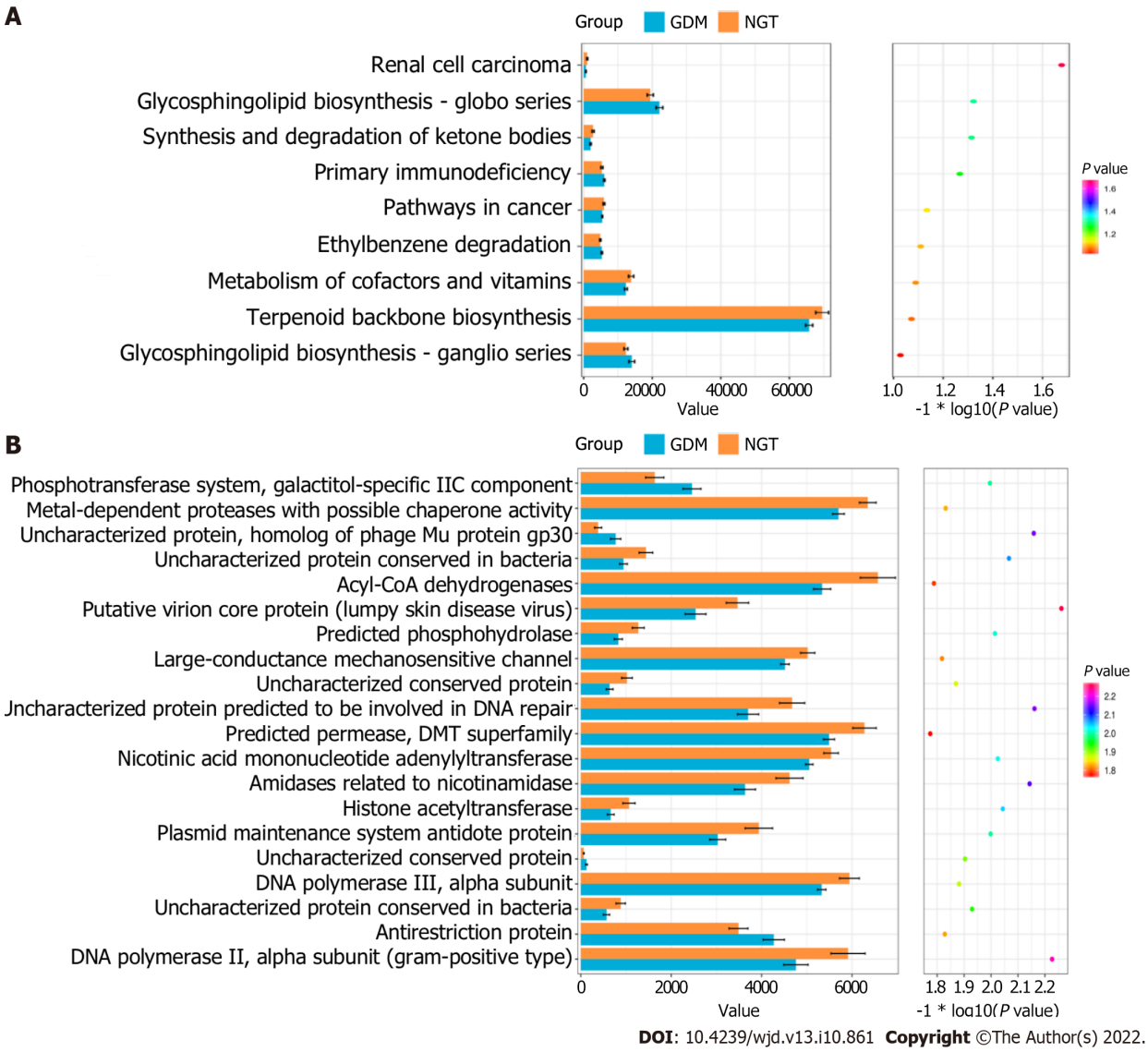

Figure 6 KEGG pathways and COG categories between the GDM and NGT groups. A: KEGG pathway; B: COG categories. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus.

key role in the interaction between the host tissues and gut microbiome[20]. The gut microbiota in the GDM patients also exhibited a higher abundance of *Bacteroidetes*, *Fusobacteria,* and *Proteobacteria* compared to healthy subjects. Proteobacteria is an opportunistic pathogen that creates a major structural imbalance in the gut microbiota of GDM patients. The ratio of *Firmicutes* to *Bacteroidetes* in GDM patients is lower than that of NGT individuals.

At the family level, *Atopobiaceae*, *Eggerthellaceae*, *Streptococcaceae*, *Christensenellaceae*, *Clostridiaceae*, *Bifidobacteriaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were more abundant in the NGT subjects than in the GDM patients. Zhang *et al*[21] reported that *Ruminococcaceae*, *Bifidobacteriaceae*, *Christensenellaceae*, *Erysipelotrichaceae*, *Peptostreptococcaceae*, and *Eggerthellaceae* were more abundant in the NGT subjects, which is consistent with the current study. In line with the study of Ma *et al*[22], the current results revealed that *Ruminococcaceae* were more abundant in the NGT group than in the GDM group. However, other studies have observed the opposite result[23,24]. The mechanisms remain unclear.

At the genus level, *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Kuang *et al*[25] found that the proportion of *Bifidobacterium* in the gut microbiota of GDM pregnant women was significantly reduced, while the proportions of *Bacteroides* and *Klebsiella* were significantly increased. Liu *et al*[26] found that compared with normal pregnant women, the proportion of *Faecalis* in GDM patients was significantly lower, while the proportion of *Prevotella* was significantly higher. In the present study, there were no significant differences in *Bifidobacterium*, *Klebsiella*, or *Prevotella* between the GDM group and NGT group. Ma *et al* found that *Ruminococcaceae_UCG-002* and *Ruminococcaceae_UCG-005* were reduced in women with GDM[22].

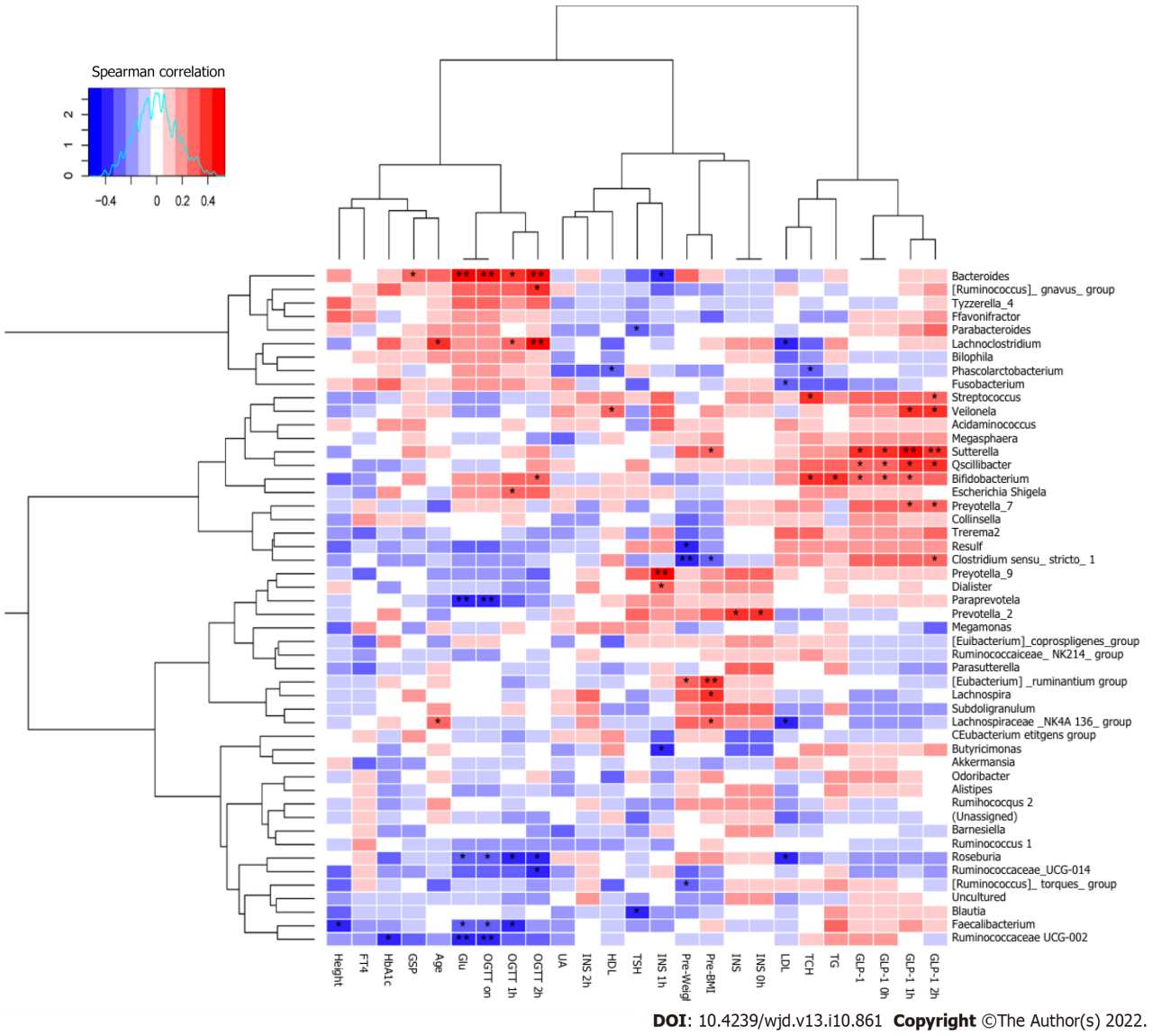

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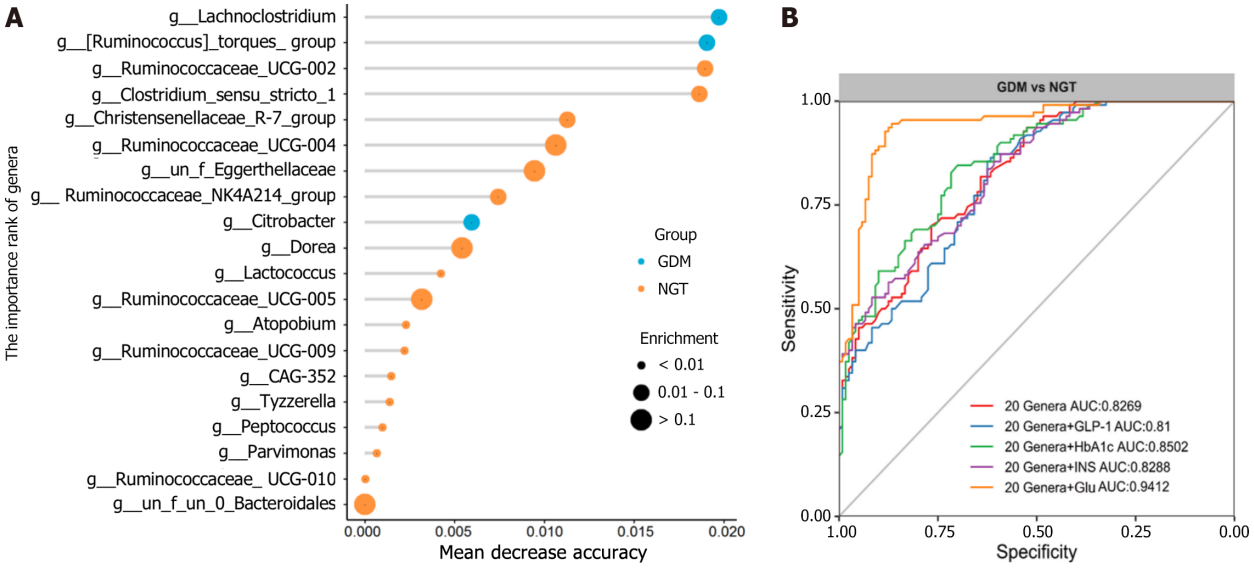
Figure 7 Spearman's correlations between different dominant genera and blood biochemical traits. [a]*P* < 0.05, [b]*P* < 0.01. GDM: Gestational diabetes mellitus; GLP-1: Glucagon-like peptide-1; GSP: Glycosylated serum protein; Glu: Glucose; HbA1c: Hemoglobin A1c; UA: Uric acid; TCH: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; INS: Insulin; OGTT: Oral glucose tolerance test; TSH: Thyroid-stimulating hormone; FT4: Free tetraiodothyronine.

One study found that supplementation with *Lactobacillus rhamnosus* in pregnant women may reduce the prevalence of GDM[27]. Another study showed that additional probiotic supplementation from pregnancy to 12 months post-delivery can reduce insulin levels and improve insulin sensitivity[28]. In this study, to identify beneficial bacteria for pregnant women, Spearman's correlation analysis was performed to identify the relationships between bacterial genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were negatively correlated with Glu. *Ruminococcaceae_UCG-002* was negatively correlated with HbA1c. *Clostridium_sensu_stricto_1*, *Desulfovibrio*, and (*Ruminococcus*)*_torques_group* were negatively correlated with pre-pregnancy body weight. *Phascolarctobacterium* was negatively correlated with HDL. *Ruminococcaceae_UCG-003* and *Faecalibacterium* were negatively correlated with height. *Lachnoclostridium* and *Lachnospiraceae_NK4A136_group* were positively correlated with age. Zhang *et al*[21] found that *Ruminococcaceae_UCG-002* was negatively correlated with fasting blood glucose levels. In the study of Crusell, *Clostridium (sensu stricto)* was positively correlated with gestational weight[29]. To the best of our knowledge, no studies have yet reported on the relationships between *Paraprevotella*, *Roseburia*, and *Faecalibacterium* and Glu in GDM. The current findings suggest that these genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment.

GLP-1 and its receptor agonist can promote insulin secretion only when the blood glucose level is elevated[30]. This safety feature makes GLP-1 and its agonist suitable for the treatment of GDM, which requires strict maintenance of blood glucose levels and stable, safe blood glucose regulation. Thus, the current study aimed to examine the correlations between the gut microbiota and GLP-1 levels, and



DOI: 10.4239/wjd.v13.i10.861

Figure 8 Gut microbiota–based prediction of gestational diabetes mellitus. A: Identification of gestational diabetes mellitus (GDM) markers by random forest models; B: Receiver operating characteristic (ROC) curves of operational taxonomic units-based diagnostic biomarkers for GDM. NGT: Normal glucose tolerance; GDM: Gestational diabetes mellitus; INS: Insulin; Glu: Glucose; HbA1c: Hemoglobin A1c; GLP-1: Glucagon-like peptide-1.

identify beneficial bacteria that can improve the expression of GLP-1 in patients with GDM, so as to more safely control blood glucose. In the present study, *Sutterella* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), GLP-1 (2 h), and pre-pregnancy BMI. Wang *et al*[31] reported that subjects taking metformin exhibited a significantly increased relative abundance of *Sutterella*, whereas liraglutide dosing was associated with a significant increase in the genus *Akkermansia*. Another study showed that *Sutterella* was associated with C-reactive protein levels[32]. In the current study, *Oscillibacter* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), and GLP-1 (2 h). One study reported that *Cyclocarya paliurus polysaccharides* alleviated type 2 diabetic symptoms by increasing eleven SCFA-producing species, including *Oscillibacter_valericigenes* and *Oscillibacter_ruminantium*[33]. *Oscillibacter* belongs to the Clostridia class of Firmicutes, and in the human gut microbiota, this bacterium grow fermentatively, predominantly producing valerate when grown using glucose as a carbon source[34]. In the current study, *Bifidobacterium* was significantly positively correlated with GLP-1 (0 h), GLP-1 (1 h), TG, TCH, and OGTT (2 h). In the study of Zhao *et al*[35], *Bifidobacterium longum DD98* improved the serum and intestinal cell GLP-1 levels, which protected pancreatic β-islet cells from damage induced by type 2 diabetes. To the best of our knowledge, this is the first report on the associations between GLP-1 and genera such as *Sutterella*, *Oscillibacter*, and *Bifidobacterium* in GDM.

CONCLUSION

In summary, this study contributes to a better understanding of the relationships between the gut microbiota and blood biochemical traits, particularly GLP-1, in individuals with GDM. The current findings suggest that some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM. Future studies combining metagenomics and metabonomics would be of value for improving our understanding of the roles of specific strains and metabolites in patients with GDM and supporting precise prevention and intervention strategies for GDM.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) places both the mother and offspring at high risk of complications. Increasing evidence suggests that the gut microbiota plays a role in the pathogenesis of GDM.

Research motivation
To confirm whether the gut microbiota is related to blood biochemical traits, particularly glucagon-like peptide-1 (GLP-1), in GDM patients.

Research objectives
To explore the correlation between the gut microbiota and blood biochemical traits.

Research methods
The V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene was sequenced based on the fecal samples of 35 pregnant women with GDM and was compared to that of 25 pregnant women with normal glucose tolerance (NGT).

Research results
The results showed that *Ruminococcaceae_UCG-002*, *Ruminococcaceae_UCG-005*, *Clostridium_sensu_stricto_1*, and *Streptococcus* were more abundant in the NGT group than in the GDM group. *Bacteroides* and *Lachnoclostridium* were more abundant in the GDM group than in the NGT group. Spearman's correlation analysis was performed to identify the relationships between bacterial genera and blood biochemical traits. *Paraprevotella*, *Roseburia*, *Faecalibacterium*, and *Ruminococcaceae_UCG-002* were significantly negatively correlated with glucose. *Ruminococcaceae_UCG-002* was significantly negatively correlated with HbA1c. *Bacteroides* was significantly positively correlated with glucose. *Sutterella*, *Oscillibacter*, and *Bifidobacterium* were significantly positively correlated with GLP-1. A random forest model showed that 20 specific genera plus glucose provided the best discriminatory power, as indicated by the area under the receiver operating characteristic curve (0.94).

Research conclusions
The results of this study reveal novel relationships between the gut microbiome, blood biochemical traits, particularly GLP-1, and GDM status.

Research perspectives
These findings suggest some genera are crucial for controlling blood glucose-related indices and may be beneficial for GDM treatment. Alteration in the microbial composition of the gut may potentially serve as a marker for identifying individuals at risk of GDM.

FOOTNOTES

Author contributions: Liang YY, Liu LY, and Jia Y designed the study, collected the samples, compiled the data, and drafted and critically revised the manuscript; Li Y, Cai JN, and Shu Y compiled the data and performed the statistical analysis and data interpretation; Tan JY collected the samples and compiled the data; Chen PY critically revised the manuscript; Cai HH and Cai XS designed this study, collected the samples, and drafted and critically revised the manuscript; all authors contributed to the article and approved the submitted version.

Supported by the National Natural Science Foundation of China, No. 81701396; Foshan Science and Technology Planning Project, No.1920001001163; and Shenzhen Guangming District Soft Science Research Project, No. 2021R01002.

Institutional review board statement: The study was approved and carried out in accordance with the guidelines of the Ethics Committee of Nanhai District People's Hospital of Foshan.

Conflict-of-interest statement: The authors declare that no competing interest exists.

Data sharing statement: Illumina sequencing reads were uploaded to the SRA under accession number PRJNA11381. The rest of the data that support the conclusions of this study are available from the corresponding author upon request.

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S-Editor: Chen YL
L-Editor: Wang TQ
P-Editor: Chen YX

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 877-887

DOI: 10.4239/wjd.v13.i10.877

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Clinical Trials Study

Effectiveness and safety of human umbilical cord-mesenchymal stem cells for treating type 2 diabetes mellitus

Xiao-Fen Lian, Dong-Hui Lu, Hong-Li Liu, Yan-Jing Liu, Xiu-Qun Han, Yang Yang, Yuan Lin, Qing-Xiang Zeng, Zheng-Jie Huang, Feng Xie, Cai-Hao Huang, Hong-Mei Wu, Ai-Mei Long, Ling-Ping Deng, Fan Zhang

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bayoumi RAL, United Arab Emirates; Salim A, Pakistan

Received: June 6, 2022
Peer-review started: June 6, 2022
First decision: August 7, 2022
Revised: August 19, 2022
Accepted: September 8, 2022
Article in press: September 8, 2022
Published online: October 15, 2022

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Abstract

BACKGROUND

Progressive pancreatic β-cell dysfunction is a fundamental part of the pathology of type 2 diabetes mellitus (T2DM). Cellular therapies offer novel opportunities for the treatment of T2DM to improve the function of islet β-cells.

AIM

To evaluate the effectiveness and safety of human umbilical cord-mesenchymal stem cell (hUC-MSC) infusion in T2DM treatment.

METHODS

Sixteen patients were enrolled and received 1 × 10^6 cells/kg per week for 3 wk as intravenous hUC-MSC infusion. The effectiveness was evaluated by assessing fasting blood glucose, C-peptide, normal glycosylated hemoglobin A1c (HbA1c), insulin resistance index (homeostatic model assessment for insulin resistance), and islet β-cell function (homeostasis model assessment of β-cell function). The dosage of hypoglycemic agents and safety were evaluated by monitoring the occurrence of any adverse events (AEs).

RESULTS

During the entire intervention period, the fasting plasma glucose level was significantly reduced [baseline: 9.3400 (8.3575, 11.7725), day 14 ± 3: 6.5200 (5.2200, 8.6900); $P < 0.01$]. The HbA1c level was significantly reduced on day 84 ± 3 [baseline: 7.8000 (7.5250, 8.6750), day 84 ± 3: 7.150 (6.600, 7.925); $P < 0.01$]. The patients' islet β-cell function was significantly improved on day 28 ± 3 of intervention [baseline: 29.90 (16.43, 37.40), day 28 ± 3: 40.97 (19.27, 56.36); $P < 0.01$]. The dosage of hypoglycemic agents was reduced in all patients, of whom 6 (50%) had a decrement of more than 50% and 1 (6.25%) discontinued the hypoglycemic agents. Four patients had transient fever, which occurred within 24 h after the second or third infusion. One patient (2.08%) had asymptomatic nocturnal hypoglycemia after infusion on day 28 ± 3. No liver damage or other side effects were reported.

CONCLUSION

The results of this study suggest that hUC-MSC infusion can improve glycemia, restore islet β-cell function, and reduce the dosage of hypoglycemic agents without serious AEs. Thus, hUC-MSC infusion may be a novel option for the treatment of T2DM.

Key Words: Type 2 diabetes; Human umbilical cord mesenchymal stem cells; Blood glucose; Homeostasis model assessment of β-cell function; Hypoglycemic agents

Core Tip: Our article focused on the effectiveness and safety of human umbilical cord mesenchymal stem cell (hUC-MSC) infusion for treating type 2 diabetes. The results suggest that hUC-MSC infusion can improve glycemia, restore islet βcell function, and reduce the dosage of hypoglycemic agents without serious adverse events.

Citation: Lian XF, Lu DH, Liu HL, Liu YJ, Han XQ, Yang Y, Lin Y, Zeng QX, Huang ZJ, Xie F, Huang CH, Wu HM, Long AM, Deng LP, Zhang F. Effectiveness and safety of human umbilical cord-mesenchymal stem cells for treating type 2 diabetes mellitus. *World J Diabetes* 2022; 13(10): 877-887
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/877.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.877

INTRODUCTION

Diabetes has been a major public health problem worldwide in recent decades. Data from the International Diabetes Federation shows that the prevalence of diabetes among adults is 463 million globally. The estimated prevalence of diabetes and prediabetes among adults in China is 10.9% and 35.7% respectively[1], of which type 2 diabetes mellitus (T2DM) accounts for more than 90% of cases. In China, only 5.6% of T2DM patients achieved glycemic control in 2017[2].

T2DM is regarded as a chronic, progressive disease that arises from an impairment in the insulin-sensing mechanisms and culminates in insulin resistance (IR). Initially, the IR is compensated by increased insulin production; however, as the T2DM progresses over time, the general pancreatic dysfunction leads to increasingly lower insulin production. As glucose continues to accumulate in the bloodstream, chronic hyperglycemia promotes a chronic vicious cycle of metabolic decline[3]. In the first 10 years of T2DM, the β-cell function reduces by ~10%, but this is followed by a period of much more rapid decrease, of an additional ~10% every 2 years, until it eventually results in insulin-dependent diabetes[4].

Current treatments for diabetes include diet control, physical exercise, oral antidiabetic agents, and insulin therapy. Although novel medications and diet therapies continue to be developed, none has provided full protection against deterioration of β-cell function[5,6]. Islet/pancreas transplantation is an efficient way to restore islet β-cell function, but its clinical application is greatly restricted by the limited resource of donor tissues or organs, the immune rejection response, and the high cost and side effects of immunosuppressive drugs[7]. Therefore, the need for an effective and safe strategy to restore β-cell function in T2DM patients remains unmet.

In recent years, mesenchymal stem cell (MSC) therapy for diabetic patients has been extensively studied[8-10] as a novel therapeutic option for diabetes. MSCs are a population of multipotent stem cells from the mesoderm. Human umbilical cord-MSCs (hUC-MSCs) have been an important resource in clinical applications with many advantages including convenient material obtainability, less ethical controversy, great differentiation potential, robust multiplication capacity, low immunogenicity, and

less chance of virus infection. The transplantation of bone marrow MSCs reduced fasting blood glucose (FBG) and significantly increased serum C-peptide (CP) level in a macaque model study[11]. Liu *et al* [12] showed that injection of UC-MSCs with a 5-d interval decreased glycosylated hemoglobin A1c (HbA1c) levels and required insulin dose in patients with T2DM. In a relatively small T2DM patient study (n = 18), Kong *et al*[13] showed responsiveness to treatment of intravenous transfusion of UC-MSCs three times with 2-wk intervals, administered over a 6-mo period. Finally, in another small-size T2DM patient study (n = 6), Guan *et al*[14] showed that treatment with intravenous transfusion of UC-MSCs two times with 2-wk intervals led to one-half of the patients becoming insulin-free between treatment months 25 and 43.

We hypothesized that hUC-MSCs restore β-cell function by differentiating into β-cells. Animal studies have previously shown that the hUC-MSCs are able to restore β-cell function and insulin secretion in diabetic rats by differentiating into islet-like cells[15,16]. Later studies showed that the transplanted hUC-MSCs were also able to reduce IR by improving the microenvironment[17,18]. However, the effectiveness and safety of hUC-MSCs in clinical application have not been fully assessed, especially in a standardized clinical study for T2DM. To explore the therapeutic effectiveness and mechanism of hUC-MSC infusion, we conducted the present study to evaluate the effectiveness and safety of hUC-MSC infusion in treating T2DM. This is the first clinical trial of hUC-MSC infusion for T2DM treatment approved by the China Medical Biotech Association.

MATERIALS AND METHODS

Patients

The enrolled participants were patients admitted to Peking University Shenzhen Hospital (Shenzhen, China) for T2DM, and all provided signed informed consent. The study was conducted according to the Declaration of Helsinki and approved by the institutional review board of Peking University Shenzhen Hospital (IRB Approval No. [2018] 29th). The patient inclusion criteria were diagnosis with T2DM[19], age between 18 years and 70 years, and HbA1c level between 7% and 9.5% during the screening and follow-up periods. There were no restrictions on treatment of the T2DM patients. The exclusion criteria were: positivity for glutamic acid decarboxylase autoantibody; treatment with thiazolidinediones within 3 mo; history of severe drug allergy; neurological deficiency induced by severe brain injury; severe respiratory disease; severe cardiovascular disease (systolic blood pressure 180 mmHg and/or diastolic blood pressure 110 mmHg or refractory hypertension); severe hepatic dysfunction or uremia; other complications of uncontrollable diabetes, such as stage V and VI diabetic retinopathy and sustained hyperglycemia or catastrophic fluctuations; endocrine and metabolic disease other than diabetes; severe hematologic disease; any acute or chronic infection; any malignancies; human immunodeficiency virus infection; severe psychiatric disease; pregnancy, planned pregnancy, or lactation; taking drugs that affect glucose metabolism within 1 mo, such as glucocorticoid, thiazide diuretic, oral contraceptive, and tricyclic antidepressant; alcohol and drug abuse; participants of any other clinical trials within 3 mo; and, any other disease or status that may influence the patient's safety or adherence according to the investigators' assessment.

hUC-MSC preparation

The hUC-MSCs were provided by Beike Biotechnology (Shenzhen, China), and the preparation was performed as previously reported[20]. The prepared hUC-MSCs were analyzed for quality according to the standards of the International Society for Cellular Therapy and stored at -196 °C[21]. Briefly, the cells were adherent to plastic, positive for cluster of differentiation (CD) 105, CD73, and CD90, and negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and human leucocyte antigen DR. The hUC-MSCs were processed according to the workflow of Peking University Shenzhen Hospital.

Study design

Upon study enrollment, all participants were assessed for diabetes, complications, diet, and exercise in the Diabetic Out-Patient Clinic over a period of 16 wk prior to the initiation of intervention. The participants were recommended a daily diet that did not exceed 25-30 kcal/kg body weight and an exercise routine composed of walking or similar exercise for 30 min three times per week; these recommendations were provided throughout the study and followup periods. By the time of initiation of hUC-MSC therapy, all patients had already accepted treatments based upon diet, exercise, and prescribed medication (oral hypoglycemic agents and insulin injections); the latter had been administered as a baseline, at stable doses for at least 2 mo (day -56 ± 3 to day 0 ± 3).

During the follow-up period, the participants performed self-monitoring of their fasting plasma glucose (FPG) and 2-h postprandial plasma glucose (P2PG) 7 times per week. The dosages of oral hypoglycemic agents and insulin were adjusted according to the patient's blood glucose to keep the level stable, at FPG range of 79.2-126 mg/dL and P2PG range of 79.2-180 mg/dL. If the total daily insulin dose was ≤ 0.2 U/kg at any time during the study period, the administration of exogenous insulin was withdrawn; if the level of blood glucose was stable with the lowest dose of a single oral

hypoglycemic drug, the oral hypoglycemic drug was withdrawn.

All patients were assessed again after 16 wk and were administered hUC-MSC infusions. The infusion was administered at a dosage of 1 × 10^6 cells/kg per week for 3 wk. Considering the possible accidental episodes in the real-life that may interrupt the patients' follow-up visits plan in due time, we set a flexible time range (± 3 d) at the patient's discretion but which would not affect the safety and effectiveness of the study. This flexible schedule was structured for in-clinic evaluations to occur on day 14 ± 3, day 21 ± 3, day 28 ± 3 and day 84 ± 3 after the first dosage (Figure 1).

Effectiveness assessment

The effectiveness assessments were performed on day 14 ± 3, 21 ± 3, 28 ± 3, and 84 ± 3, including FBG, 2-h postprandial blood glucose, HbA1c, fasting CP (FCP), 2-h postprandial CP (P2CP), IR index [homeostatic model assessment for IR (HOMA-IR)] (by CP) = 1.5 + FCP ´ FBG/2800, islet β-cell function [HOMA of β-cell function (HOMA-β)] (by CP) = 0.27 ´ FCP/(FBG - 3.5), and hypoglycemic agent dosage. These dosages were adjusted by the treating physician according to standard clinical practice, based on blood glucose and A1c results.

Safety assessment

Any adverse event after receiving the hUC-MSC infusions would be reported and recorded. Routine safety assessment was conducted according to the visit schedule, including blood routine examination, hepatic function test, electrocardiogram, chest radiography, and tumor-associated antigen test.

Statistical analyses

All statistical analyses were analyzed with SPSS® 25.0 software (IBM Corp, Armonk, NY, United States). Quantitative data were analyzed with the two-samples Wilcoxon test. Differences in proportions were analyzed by the two-tailed test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 16 T2DM patients were enrolled. The clinical characteristics are shown in Table 1.

Intravenous infusion of hUC-MSCs significantly improved FG

FPG was significantly reduced on day 14 ± 3 without any alteration of hypoglycemic drug dosage, achieving the lowest level during the entire intervention period [baseline: 9.3400 (8.3575, 11.7725), day 14 ± 3: 6.5200 (5.2200, 8.6900); $P < 0.01$]. The FBG had a sustained decrease during the follow-up visit period, with a reduction of hypoglycemic agents for all patients. HbA1c level was significantly reduced on day 84 ± 3 [baseline: 7.8000 (7.5250, 8.6750), day 84 ± 3: 7.150 (6.600, 7.925); $P < 0.01$] (Figure 2). There was no significant difference in postprandial blood glucose level in the 75-g oral glucose tolerance test without hypoglycemic agents.

Intravenous infusion of hUC-MSCs improved islet β-cell function

The patients' islet β-cell function was significantly improved on day 28 ± 3 [baseline: 29.90 (16.43, 37.40), day 28 ± 3: 40.97 (19.27, 56.36); $P < 0.01$]. Islet β-cell function (HOMA-β) improvement was stably sustained during the following intervention period, with a reduction in hypoglycemic agents in all patients. The HOMA-IR decreased but not to a level that was statistically significant (Figure 2).

Intravenous infusion of hUC-MSCs decreased the dosage of hypoglycemic agents

After intravenous infusion of hUC-MSCs, the dosage of hypoglycemic agent was reduced in all patients on day 28 ± 3, of whom 12 (75%) had a decrement of 10%-50% and 4 (25%) had a decrement of 50%. On day 84 ± 3, the dosage was reduced in all patients, of whom 6 (50%) had a decrement of more than 50% and 1 (6.25%) discontinued the hypoglycemic agents (Figure 2).

Safety assessment

Four patients had transient fever (5 times in total), which occurred within 24 h after the second or third infusion. One patient (2.08%) had asymptomatic nocturnal hypoglycemia after infusion on day 28 ± 3. It did not recur after reducing the dosage of insulin in the following period. No patient had acute diabetic complications during the intervention period.

The leukocytes transiently increased significantly on day 14 ± 3 after the first dosage of hUC-MSCs, but there was no significant difference in leukocytes after the second dosage compared with baseline. The leukocyte level remained stable in the following period. There were no significant alterations in serum levels of alanine aminotransferase and creatinine (Figure 3).

Table 1 Baseline clinical characteristics of the patients

Characteristic	*n* = 16
Age (yr)	52.5 ± 7.91
Male	12
Female	4
Duration of diabetes (yr)	10.06 ± 5.74
BMI (kg/m^2)	24.47 ± 2.76
Glucose (mmol/L)	
FPG	9.66 ± 2.65
P2PG	16.32 ± 4.64
HbA1c (%)	8.01 ± 0.63
C peptide (pmol/L)	
FCP	741.56 ± 464.50
P2CP	1596.70 ± 989.65
HOMA-IR	4.22 ± 1.91
HOMA-β (%)	35.01 ± 24.35

BMI: Body mass index; FPG: Fasting plasma glucose; P2PG: 2-h postprandial plasma glucose; HbA1c: Glycosylated hemoglobin; FCP: Fasting c-peptide; P2CP: 2-h postprandial c-peptide; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-β: Homeostasis model assessment of β-cell function.

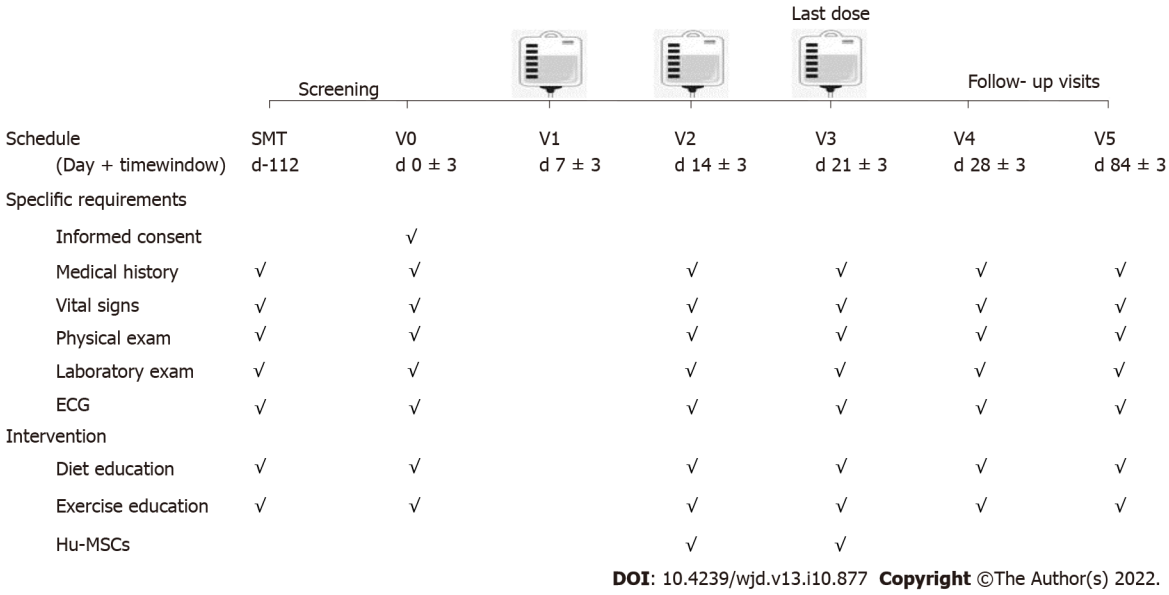
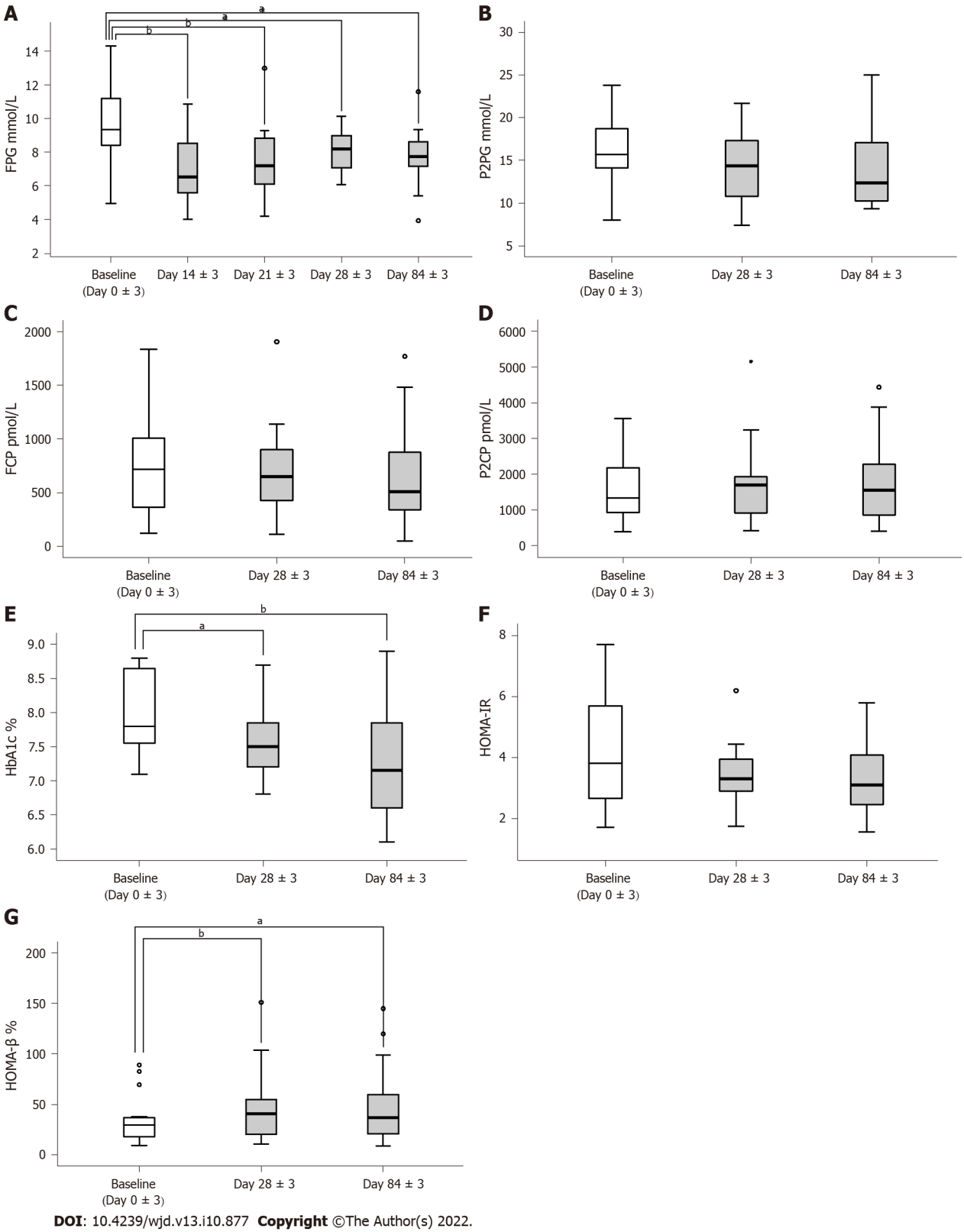


Figure 1 Flow chart for the study procedure. The patients enrolled in the present study received three infusions on days 7 ± 3, 14 ± 3, and 21 ± 3. There were three visits, on days 0 ± 3, 28 ± 3, and 84 ± 3. ECG: Electrocardiogram; hUC-MSCs: Human umbilical cord blood-mesenchymal stem cells.

After three doses of hUC-MSCs, carbohydrate antigen 199 and alpha fetoprotein decreased on day 28 ± 3. There was no significant alteration in carcinoembryonic antigen (Figure 3) or pancreatic autoantibody in the patients. All patients were negative for islet autoantibodies, with the exception of 1 who was positive for anti-islet cell antibody before and after the intervention.

DISCUSSION

Previous studies have demonstrated that hUC-MSCs are capable of decreasing the levels of FPG and



DOI: 10.4239/wjd.v13.i10.877

Figure 2 Assessment of the effectiveness of human umbilical cord blood-mesenchymal stem cell treatment. A: Fasting plasma glucose tested on day 0 ± 3, day 14 ± 3, day 21 ± 3, day 28 ± 3, and day 84 ± 3; B-D: 2-h postprandial blood glucose, fasting C-peptide, and 2-h postprandial C-peptide tested on day 0 ± 3, day 28 ± 3, and day 84 ± 3; E: Glycosylated hemoglobin A1c levels tested on day 0 ± 3, day 28 ± 3, and day 84 ± 3; F and G: Homeostasis model assessment of insulin resistance and homeostasis model assessment of β-cell function calculated on day 0 ± 3, day 28 ± 3, and day 84 ± 3. $^{a}P < 0.05$, $^{b}P < 0.01$. FPG: Fasting plasma glucose; P2BG: 2-h postprandial blood glucose; FCP: Fasting C-peptide; P2CP: 2-h postprandial C-peptide; HbA1c: Glycosylated hemoglobin A1c; HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-β: Homeostasis model assessment of β-cell function.

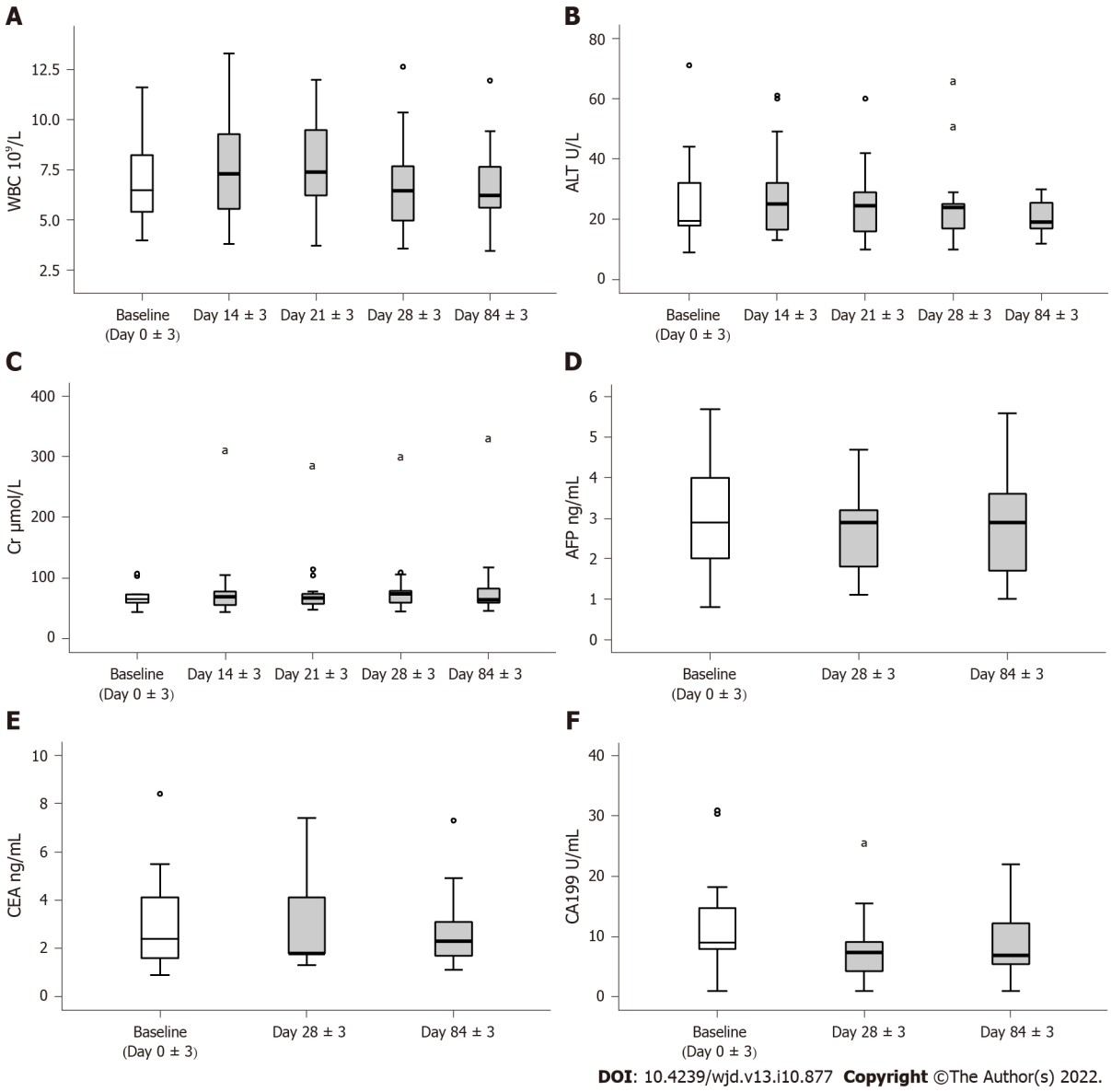


Figure 3 Assessment of the safety of human umbilical cord blood-mesenchymal stem cell treatment. A-C: Leukocytes, hepatic and renal function; D-F: Antigen associated with tumor. [a]*P* < 0.05.

HbA1c and reducing the dosage of hypoglycemic agents[13,22-24]. FBG was decreased and islet β-cell function was significantly improved after treatment with hUC-MSCs in our preliminary study in a diabetic rat model (data not shown). The purpose of this study was to evaluate the effectiveness and safety of hUC-MSC intravenous infusion in the short term for T2DM patients. The results demonstrated that hUC-MSCs could ameliorate hyperglycemia by decreasing FPG and HbA1c and reducing the dosage of hypoglycemic agents. It also improved islet β-cell function. Bell *et al*[25] observed the expression of pancreatic and duodenal homeobox 1 and islet cell differentiation gradually increased after hUC-MSC treatment of streptozotocin-treated NOD-SCID mice. MSC transplantation in streptozotocin-treated mice promoted the proliferation of endogenous islet cells and increased the amount of islet β-cells and insulin secretion[26]. Si *et al*[27] showed that the "increased" pancreatic islets and islet β-cells were not due to cell proliferation but to tissue repair and a decrease in apoptosis and damage in a rat model of T2D. Caplan *et al*[28] demonstrated that MSCs could ameliorate β-cell damage and restore islet β-cell function in a murine model in the early stage (7 d) of MSC treatment. Liu *et al*[12] reported that Wharton's jelly-derived MSC transplantation increased the HOMA-β from 65.99 ± 23.49 % to 98.86 ± 43.91%. The clinical trial results of Hu *et al*[22] also showed that the HOMA-β was significantly increased 1 mo after hUC-MSC treatment and was maintained for 18 mo. The results of the current study were consistent with these findings, indicating that improvement in glycemia in T2DM patients after hUC-MSC treatment was related to the repair of islet β-cells.

IR plays a critical role in the development of T2DM. It has been reported that MSC treatment in the early stage improved IR in animal experiments[27]. Chen *et al*[24] showed that hUC-MSC treatment could increase the area under curve of the CP and decrease the HOMA-IR. Hu *et al*[22] showed that

hUC-MSC treatment decreased the FCP and improved the HOMA-IR. However, the present study found no significant improvement of IR and no significant decrease in FCP and P2CP during the follow-up period. The islet β-cell function and IR state of patients in this study will be extensively followed for further analysis to elucidate the mechanisms underlying glycemia improvement.

Embryonic stem cells have the risk of teratoma formation, which limits their clinical application[29]. While the MSCs have been documented as having therapeutic efficacy for inflammation-related diseases, the concerns of possible tumorigenic effects are undeniable; although some studies have shown that MSCs do not undergo malignant transformation[30,31]. Guan *et al*[14] observed no immediate or delayed toxicity associated with MSC administration (within the followup period). In the present study, we observed no significant alterations in tumor-associated antigens (alpha-fetoprotein, carcinoembryonic antigen, carbohydrate antigen 199) within the follow-up period. Because the follow-up time was short, we plan to follow up the participants for 3 years for further observations of possible transplant complications.

As this was a preliminary exploratory study, our sample size was limited; we plan to recruit more participants and include a healthy control group in our future study to evaluate the clinical utility of this therapy for T2DM.

CONCLUSION

The results of our study suggest that hUC-MSC treatment can improve glycemia, restore islet βcell function, and safely reduce the dosage of hypoglycemic agents required by the patient. Thus, hUC-MSC treatment could be a novel therapy for T2DM.

ARTICLE HIGHLIGHTS

Research background

Cellular therapies offer novel opportunities for the treatment of type 2 diabetes mellitus (T2DM) to improve the function of islet β-cells. However, the effectiveness and safety of human umbilical cord-mesenchymal stem cell (hUC-MSCs) in clinical application have not been fully assessed.

Research motivation

We conducted the present trial to explore the therapeutic effectiveness and mechanism of hUC-MSC infusion for treating T2DM.

Research objectives

We hypothesized that hUC-MSCs restore β-cell function by differentiating into β-cells. We conducted the present trial to treat T2DM with hUC-MSC infusion and evaluated the effectiveness and safety of hUC-MSC therapy.

Research methods

Patients were enrolled and received 1 × 10^6cells/kg per week for 3 wk of intravenous hUC-MSC infusion. The effectiveness was assessed by fasting blood glucose, C-peptide, normal glycosylated hemoglobin A1c level (HbA1c), insulin resistance (IR) index (homeostasis model assessment of insulin resistance), islet β-cell function (homeostasis model assessment of β-cell function), and dosage of hypoglycemic agents, and the safety was evaluated by monitoring the occurrence of any adverse events.

Research results

During the entire intervention period, the fasting plasma glucose level and HbA1c were significantly reduced. The patients' islet β-cell function was significantly improved, and the dosage of hypoglycemic agents was reduced in all patients without serious adverse events.

Research conclusions

We hypothesize that hUC-MSCs restore β-cell function by differentiating into β-cells. Our study suggests that hUC-MSC treatment can improve glycemia, restore islet βcell function, and safely reduce the dosage of hypoglycemic agents.

Research perspectives

Islet β-cell function and IR state of the patients in this study will be extensively followed for further analysis to elucidate the mechanisms underlying glycemia improvement.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from all of the funders. The authors also want to thank all patients and their families who consented to participate in the study.

FOOTNOTES

Author contributions: Zhang F designed the report; Lian XF, Lu DH, Liu HL, Liu YL, Yang Y, Lin Y, Zeng QX, Huang ZJ, Xie F, Huang CH, Wu HM, Long AM, and Deng LP collected the patient's clinical data; Lian XF and Han XQ analyzed the data and wrote the paper.

Supported by Shenzhen Science and Technology Innovation Committee Projects, No. JCYJ20170816105416349; Shenzhen High-level Hospital Construction Fund; and Shenzhen Key Medical Discipline Construction Fund, No. SZXK010.

Institutional review board statement: This study was approved by the Ethics Committee of the Ethical Committee of the Peking University Shenzhen Hospital (IRB of Peking University Shenzhen Hospital [2018] 29th).

Clinical trial registration statement: This study is registered in the Chinese Clinical Trial Registry, Registration No. ChiCTR2200057370.

Informed consent statement: The participants were enrolled from patients admitted to Peking University Shenzhen Hospital for diabetes mellitus and all had signed informed consent.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: There are no additional data.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

Country/Territory of origin: China

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 October 15; 13(10): 888-899

DOI: 10.4239/wjd.v13.i10.888

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Clinical Trials Study

Metabonomics fingerprint of volatile organic compounds in serum and urine of pregnant women with gestational diabetes mellitus

Si-Ri-Gu-Leng Sana, Guang-Min Chen, Yang Lv, Lei Guo, En-You Li

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Dąbrowski M, Poland; Jovandaric M, Serbia; Suravajhala PN, India

Received: June 18, 2022
Peer-review started: June 18, 2022
First decision: July 14, 2022
Revised: July 23, 2022
Accepted: September 12, 2022
Article in press: September 12, 2022
Published online: October 15, 2022

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Abstract

BACKGROUND

Gestational diabetes mellitus (GDM) is a metabolic disease with an increasing annual incidence rate. Our previous observational study found that pregnant women with GDM had mild cognitive decline.

AIM

To analyze the changes in metabonomics in pregnant women with GDM and explore the mechanism of cognitive function decline.

METHODS

Thirty GDM patients and 30 healthy pregnant women were analyzed. Solid-phase microextraction gas chromatography/mass spectrometry was used to detect organic matter in plasma and urine samples. Statistical analyses were conducted using principal component analysis and partial least squares discriminant analysis.

RESULTS

Differential volatile metabolites in the serum of pregnant women with GDM included hexanal, 2-octen-1-ol, and 2-propanol. Differential volatile metabolites in the urine of these women included benzene, cyclohexanone, 1-hexanol, and phenol. Among the differential metabolites, the conversion of 2-propanol to acetone may further produce methylglyoxal. Therefore, 2-propanol may be a potential marker for serum methylglyoxal.

CONCLUSION

2-propanol may be a potential volatile marker to evaluate cognitive impairment in pregnant women with GDM.

Key Words: Gestational diabetes mellitus; Gas chromatography/mass spectrometry;

Humoral biomarkers; 2-propanol

Core Tip: Gas chromatography-mass spectrometry was used in a metabonomics analysis to determine the changes in volatile metabolites in pregnant women with gestational diabetes mellitus (GDM) and to explore the mechanism of cognitive function decline in these women. 2-propanol was identified as a potential volatile marker to evaluate cognitive impairment in pregnant women with GDM.

Citation: Sana SRGL, Chen GM, Lv Y, Guo L, Li EY. Metabonomics fingerprint of volatile organic compounds in serum and urine of pregnant women with gestational diabetes mellitus. *World J Diabetes* 2022; 13(10): 888-899
URL: https://www.wjgnet.com/1948-9358/full/v13/i10/888.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i10.888

INTRODUCTION

Gestational diabetes mellitus (GDM) is a metabolic disorder in which hyperglycemia develops during pregnancy in women who did not previously have diabetes[1]. The incidence of GDM varies in different countries and regions, ranging from 1 to 30% of pregnancies, and is highest in Africa, Asia, and India [2]. Epidemiological evidence indicates a continuous increase in the incidence of GDM worldwide. The presence of hyperglycemia during gestation is often associated with various abnormalities, such as obesity, cardiovascular disease, preeclampsia, and even stillbirth. GDM diagnosed at 24 to 28 wk of gestation reportedly affects fetal development[3]. The substantial effect that GDM can have on maternal and fetal health necessitates the development of a screening method for predictive and diagnostic biomarkers of GDM in early stages of pregnancy[4].

Refinements of metabonomics research methods have led to the widespread use of the mature technology in various fields, including studies of disease mechanisms and diagnosis, treatment, treatment effects, and prevention. It is helpful to analyze the changes in metabolic substances caused by pathophysiological changes in diseases[5]. Metabonomics clinical research mainly obtains relevant differential substances by analyzing patients' serum, urine, and feces. There are many metabonomics technologies, and each has shortcomings. However, the use of the technology in combination with another technology, such as liquid/gas chromatography-mass spectrometry (LC/GC-MS), can improve their respective advantages and compensate for the shortcomings of each technology. This integration also improves the metabonomic method and can obtain more reliable clinical sample data. Sample analyses involved principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal projections to latent structures-DA. These analyses have identified differential metabolites. Bioinformatics database analysis of the relevant metabolic pathway can explain the possible metabolic mechanisms and pathophysiological changes and verify the biomarkers related to the disease mechanism.

We previously reported that patients with GDM have mild cognitive decline, but the underlying mechanism remains unclear[6]. A causative association of cognitive decline with metabolic abnormalities in patients with GDM is conceivable. To explore this speculation, we analyzed the blood and urine of GDM patients and normal pregnant women using solid-phase microextraction (SPME) GC/MS to observe abnormal metabolites in GDM and identify potential biomarkers for GDM.

MATERIALS AND METHODS

Subjects and protocol

Patients aged 18 to 35 years with American Society of Anesthesiologists physical status I-II were enrolled. Thirty women with GDM who were diagnosed, followed, and treated at the First Affiliated Hospital of Harbin Medical University were included in the study. Thirty age-matched pregnant women without diabetes constituted the normal pregnancy (NP) group. All patients and volunteers read and signed informed consent forms before enrollment in the study. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University and registered with the Chinese Clinical Trial Registry (registration number: ChiCTR2000038703).

GDM was diagnosed with at least one abnormal result during the oral glucose tolerance test: Plasma glucose during fasting ≥ 92 mg/dL (5.1 mmol/L) or ≥ 180 mg/dL (10.0 mmol/L) at 1 h or ≥ 153 mg/dL (8.5 mmol/L) at 2 h. Patients with pre-gestational type 1 (T1) or type 2 (T2) DM were not included in the

study. Patients with unnatural pregnancy or a gestational period of < 37 wk or > 41 wk were excluded. Subjects on medications affecting cognitive function, including corticosteroids, antidepressants, or antiepileptics, were also not included. Subjects with chronic metabolic, endocrine, inflammatory diseases, cancer, drug or alcohol dependency, history of major brain abnormalities (*e.g.*, tumors and hydrocephaly), epilepsy, or Parkinson's disease were excluded. The psychological status of pregnant women was assessed using the Hamilton Depression Scale; a score > 7 indicated the potential for depression and was the final exclusion criterion.

On the survey data, all the enrolled patients underwent routine medical history inquiries, physical examinations, and laboratory measurements. The clinical research coordinators used a standard questionnaire to collect information on demographic characteristics and medical history (Figure 1). All pregnant women were instructed to maintain their usual physical activity and diet for at least 3 d before the survey. After overnight fasting for ≥ 10 h, venous blood and urine samples were collected and stored at -80 °C. All parameters were measured within 6 mo of sample collection.

SPME

A 75 μM extraction head was used. The coating material was carbon molecular sieve of polydimethylsiloxane. An automatic sample injector was used for heating and extraction. A puncture made in the liquid sample bottle allowed injection. In the headspace extraction method, the extraction temperature was 40 °C, and the extraction time was 20 min. After the extraction and concentration of the samples were completed, the automatic sampling device inserted the extraction head into the GC-MS injection port for analysis.

GC/MS analysis

All analyses were performed on a model QP2010 GC/MS (Shimadzu) equipped with a DB-5MS PLOT column (length: 30 m; inner diameter: 0.250 μm; film thickness, 0.25 mm; Agilent Technologies). The injections were performed in splitless mode, with a splitless time of 1 min. The injector temperature was set at 200 °C, and helium was used as the carrier gas at a flow rate of 2 mL/min. The temperature in the column was maintained at 40 °C for 2 min to condense hydrocarbons. The temperature was then increased to 200 °C at a rate of 70 °C /min and held for 1 min. Subsequently, the temperature was increased to 230 °C at 20 °C/min and maintained for 3 min. MS analyses were performed in full-scan mode with an associated m/z range of 35–200 amu. An ionization energy of 70 eV was used for each measurement, and the ion source was maintained at 200 °C.

Statistical analysis

Statistical analyses were performed using the SIMCA-p + 11 software. Differences in volatile organic carbons (VOCs) between groups were tested using PLS-DA and PCA. SIMCA-p software was used to prevent overfitting by applying default seven-round cross-validation. Additionally, permutation tests using 200 iterations were performed to further validate the supervised model. Potential metabolic biomarkers were selected based on variable importance in the projection values calculated from the PLS-DA model. For all data analyses, $P < 0.05$ indicated statistical significance. The area under the curve (AUC) of the combined biomarkers and sensitivity and specificity calculations were performed using R language software 3.2 (R Development Core Team 2011).

RESULTS

Basic information of subjects

Sixty pregnant women participated in this study, including 30 pregnant women with GDM in the GDM group and 30 healthy pregnant women in the NP group. Body weight, blood glucose level, and hemoglobin A1c level in the GDM group were significantly higher than of those in the NP group ($P < 0.05$) (Table 1).

Blood sample analysis

Eighteen significant differential metabolites were evident between the GDM and NP groups (Table 2). Comparing the GDM and NP groups revealed a good separation trend in the two groups in the two-dimensional PCA score diagram (Figure 2A). When a single prediction component and three orthogonal components were used, the PLS-DA score map ($R^2X[1] = 0.203743$, $R^2X[2] = 0.123147$, $T^2 = 0.95$) revealed a good separation effect of the data of the GDM and NP groups (Figure 2B and C). Additionally, 200 iterations were conducted to test the supervision model. The R^2 and Q^2 values calculated from the converted data were lower than their original verification values [$R^2 = (0.0, 0.241)$, $Q^2 = (0.0, -0.269)$], which proved the effectiveness of the supervision model (Figure 2D). The receiver operating characteristic (ROC) curves showed that the AUC of the three VOCs was greater than 0.5; the closer it was to 1, the better was the diagnostic effect (Figure 2E and F).

Table 1 Demographic characteristics

	GDM	NP	*t*	*P* value
Sample	30	30		
Age (yr)	28.38 ± 2.52	29.14 ± 3.61	0.95	0.35
Height (cm)	162.34 ± 4.69	164.61 ± 5.36	1.75	0.09
Weight (kg)	76.33 ± 9.16	74.05 ± 8.97	0.98	0.33
Glucose (mmol/L)	4.825 ± 1.03	3.39 ± 0.56	6.70	< 0.001
Hba1c (%)	5.93 ± 0.73	4.86 ± 0.93	4.50	< 0.001

Data are expressed as the mean ± SD. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; HbA1c: Hemoglobin A1c.

Table 2 Differential metabolites in blood volatile organic carbons of the two groups

Differential metabolite	VIP	*P* value	Time	FC (GDM/NP)
1-Octyn-3-ol, 4-ethyl-	2.2718	0.0000	9.1501	-0.3956
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester	1.6588	0.0000	4.3833	-0.3885
Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-	2.11317	0.0000	8.6667	-0.4162
Isolongifolene-5-ol	1.85815	0.0000	18.1917	-0.6099
Oxime-, methoxy-phenyl-	1.06288	0.0001	6.3083	-0.2675
Trans-beta-Ocimene	2.01398	0.0000	6.9250	-0.3406
1H-Pyrazole-1-carbothioamide, 3,5-dimethyl-	1.66239	0.0000	4.7250	-0.5068
1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	1.38245	0.0000	18.8000	-0.9345
2-Octen-1-ol, (E)-	1.11297	0.0287	8.0417	0.5487
2-Propanol, 1,1,1-trichloro-2-methyl-	1.32854	0.0000	7.8167	-0.2366
3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-	1.51135	0.0000	18.7750	-0.5299
3-Heptanol, 5-methyl-	1.2384	0.0001	5.3235	-0.2684
5-Methyluridine, tris(trifluoroacetate)	2.04962	0.0000	10.6833	-0.2948
Hexanal	1.09786	0.0001	4.0858	0.3192
Malonic acid, bis (2-trimethylsilylethyl ester	1.59984	0.0000	19.5083	-0.3655
Oxalic acid, 2TMS derivative	1.13131	0.0097	16.6477	0.1542
Quinoxalin-2-one, decahydro-3-(3,3-dimethyl-2-oxobutenylideno)-	1.73099	0.0000	7.3270	-0.0682

VIP: Variable importance in the projection; FC: Fold change; GDM: Gestational diabetes mellitus; NP: Normal pregnancy.

Urine sample analysis

Eleven meaningfully differential metabolites were found between the GDM and NP groups (Table 3). Comparison between the GDM and NP groups revealed a good separation trend in the two-dimensional PCA score diagram (Figure 3A). When using a single prediction component and three orthogonal components, the PLS-DA score map ($R^2X[1] = 0.295851$, $R^2X[2] = 0.221649$, $T^2 = 0.95$) showed that the data from the GDM and NP groups also had a good separation effect (Figure 3B and C). Additionally, 200 iterative permutations were conducted to test the supervision model. The R^2 value and Q^2 value calculated from the converted data were lower than their original verification values [$R^2 = (0.0, 0.425)$, $Q^2 = (0.0, -0.33)$], which proved the effectiveness of the supervision model (Figure 3D). The ROC curves (Figure 3E and F) were the same as those described previously.

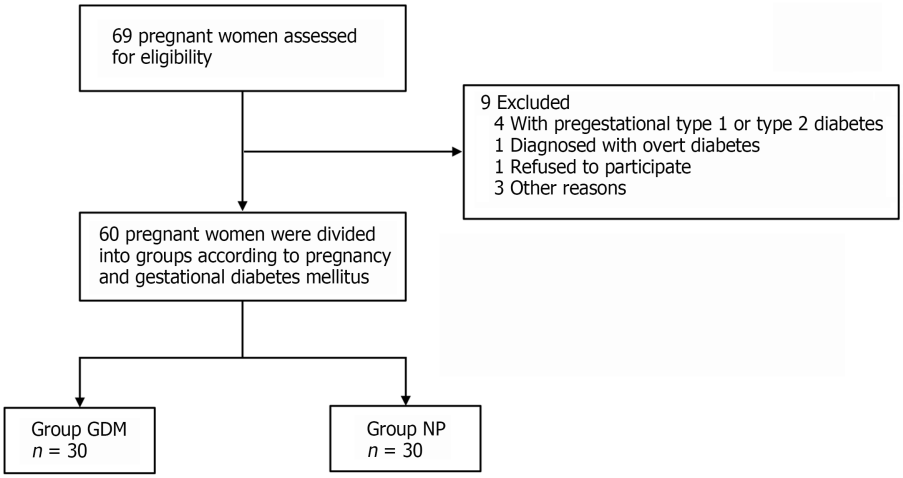
DISCUSSION

Investigations of metabolic substances in body fluids are an important supplement to the path-

Table 3 Differential metabolites in urine volatile organic carbons of the two groups

Differential metabolite	Similarity	VIP	*P* value	Time	FC (GDM/Ctrl)
Benzene, 1,3-bis(1,1-dimethylethyl)-	88	2.87589	0.0000	13.927	0.560880597
2-Pentanone	87	2.2746	0.0000	3.017	-0.077266923
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester	59	1.93204	0.0001	4.368	-0.339022989
Thiophene, 3,3'-(1,2-ethenediyl)bis-	58	1.62894	0.0000	4.371	-0.184457329
Cyclohexanone	95	1.13467	0.0081	6.018	0.935083138
.alpha.-Pinene	94	1.51705	0.0015	6.916	-0.221670672
Phenol	98	1.02127	0.0135	7.970	1.492584113
1-Hexanol, 2-ethyl-	96	1.16684	0.0444	9.118	0.693139386
Cyclododecanol	83	2.53908	0.0000	12.365	-0.41722675
3,4-Dimethylcyclohexanol	78	2.33881	0.0207	13.488	-0.094482061

VIP: Variable importance in the projection; FC: Fold change; GDM: Gestational diabetes mellitus.



DOI: 10.4239/wjd.v13.i10.888

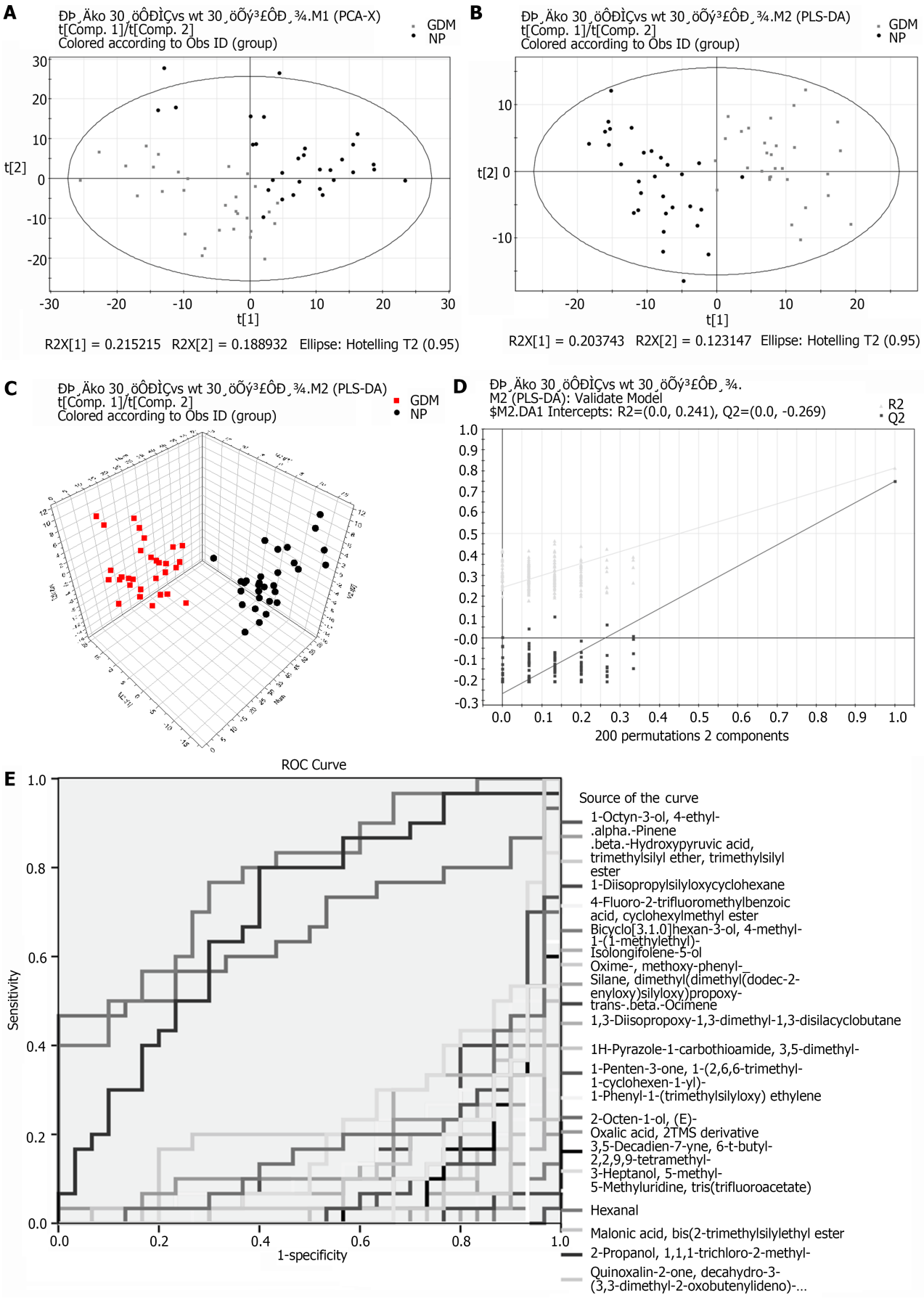
Figure 1 Patient recruitment flowchart. GDM: Gestational diabetes mellitus; NP: Normal pregnancy.

ophysiology of GDM and help identify new biomarkers and effective therapeutic targets. In this study, we observed VOCs in the blood and urine of GDM patients and compared them with age-matched normal patients. Abundant organic matter was detected in the blood and urine of GDM patients. Some of the compounds had diagnostic value.

The following five compounds were highly expressed in the GDM group: 2-octen-1-ol; 2-propanol, 1,1,1-trichloro-2-methyl; hexanal; oxalic acid, 2TMS derivative; and oxime-, methoxy-phenyl. Most of these compounds are alcohols or aldehydes.

Aldehydes are active carbonyl organic molecules that are widely present in the body. They have variable structures. The structures of over 20 types of active aldehydes have been determined and studied. These include hexanal found in this study[7]. ROC curves for hexanal correlated well with the specificity and sensitivity of GDM (AUC > 0.5). The findings implicate hexanal as a potential marker of GDM.

Active aldehydes are mainly produced during lipid and glucose metabolism (including enzymatic and non-enzymatic pathways). The enzyme pathway usually involves an aldehyde intermediate or by-product produced during glucose and lipid metabolism *in vivo*[8]. This is also consistent with the disorder of active aldehyde metabolism observed in pregnant women with GDM. Under pathological conditions, aldehyde metabolism is disordered, resulting in abundant accumulation of aldehyde and formation of an aldehyde microenvironment[9]. Aldehyde metabolism disorders are involved in the occurrence and development of various diseases. Active aldehydes are closely related to the pathogenesis of endocrine diseases. Our previous study found that serum methylglyoxal (MGO) levels in pregnant women with GDM were significantly higher than those in healthy pregnant women[6].


A
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.215215 R2X[2] = 0.188932 Ellipse: Hotelling T2 (0.95)
B
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.203743 R2X[2] = 0.123147 Ellipse: Hotelling T2 (0.95)
C
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
D
ÐÞ¸Äko 30¸öÔÐÌÇvs wt 30¸öÕý³£ÔÐ¸¾.
M2 (PLS-DA): Validate Model
$M2.DA1 Intercepts: R2=(0.0, 0.241), Q2=(0.0, -0.269)
R2
Q2
200 permutations 2 components
E
ROC Curve
Sensitivity
1-specificity
Source of the curve
1-Octyn-3-ol, 4-ethyl-
.alpha.-Pinene
.beta.-Hydroxypyruvic acid, trimethylsilyl ether, trimethylsilyl ester
1-Diisopropylsilyloxycyclohexane
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester
Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1-methylethyl)-
Isolongifolene-5-ol
Oxime-, methoxy-phenyl-_
Silane, dimethyl(dimethyl(dodec-2-enyloxy)silyloxy)propoxy-
trans-.beta.-Ocimene
1,3-Diisopropoxy-1,3-dimethyl-1,3-disilacyclobutane
1H-Pyrazole-1-carbothioamide, 3,5-dimethyl-
1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-
1-Phenyl-1-(trimethylsilyloxy) ethylene
2-Octen-1-ol, (E)-
Oxalic acid, 2TMS derivative
3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-
3-Heptanol, 5-methyl-
5-Methyluridine, tris(trifluoroacetate)
Hexanal
Malonic acid, bis(2-trimethylsilylethyl ester
2-Propanol, 1,1,1-trichloro-2-methyl-
Quinoxalin-2-one, decahydro-3-(3,3-dimethyl-2-oxobutenylideno)-...

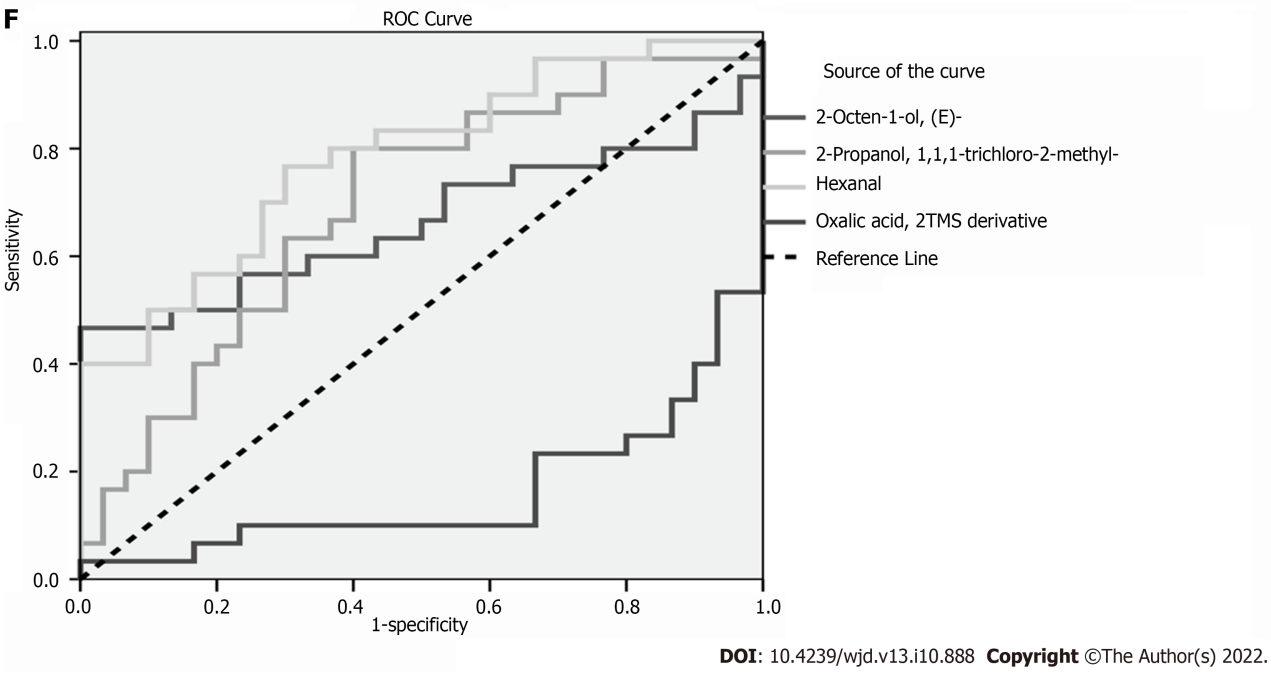


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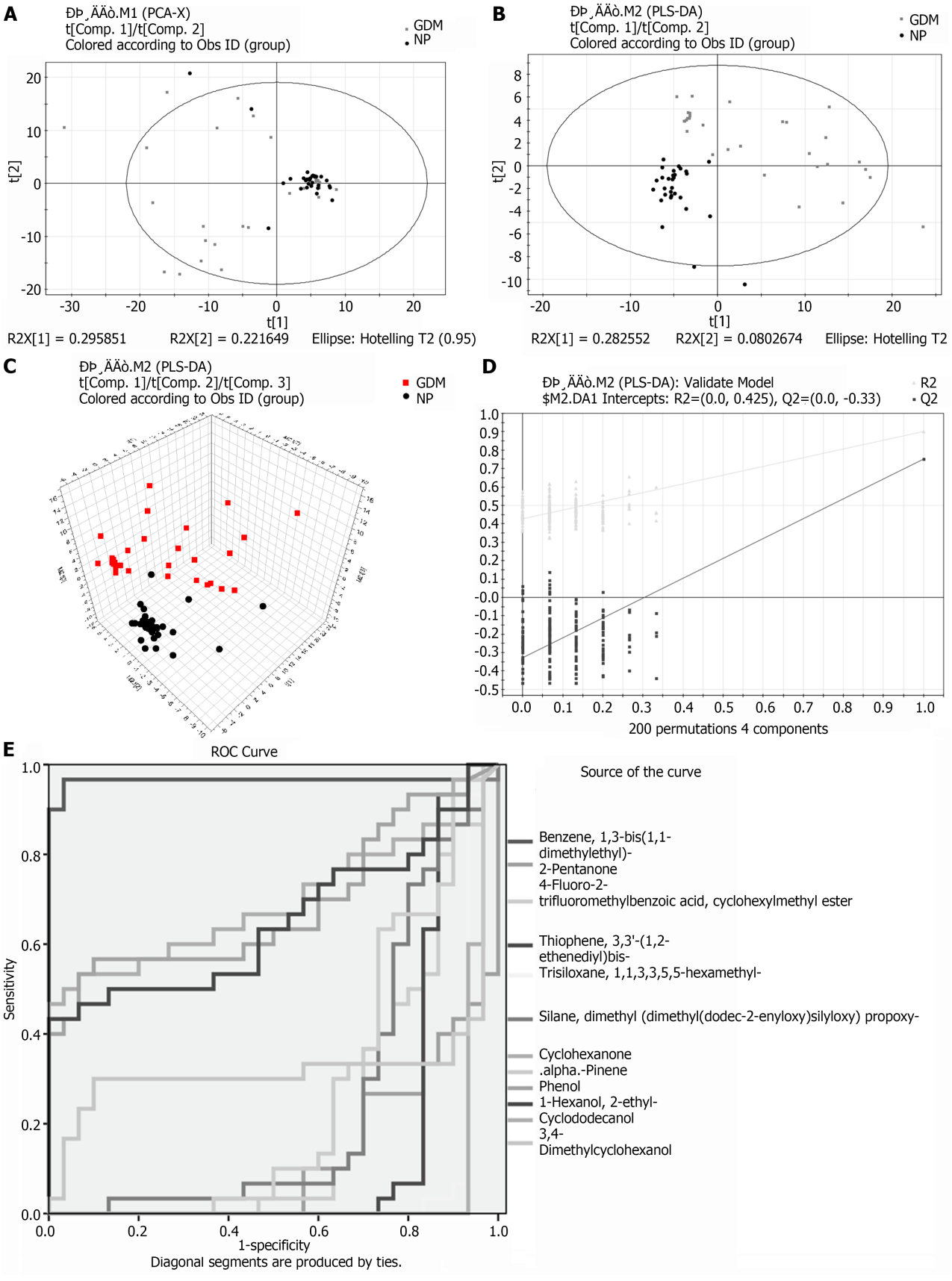
Figure 2 Metabolomic analysis of blood between groups. A: Principal component analysis score plot; B and C: Partial least squares-discriminant analysis (PLS-DA) score plots; (R^2X[1] = 0.203743, R^2X[2] = 0.123147, T^2 = 0.95); D: PLS-DA validation plot intercepts: R^2 = (0.0, 0.241), Q^2 = (0.0, -0.269); E and F: Receiver operating characteristic curves of differential substances from blood. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; ROC: Receiver operating characteristic.

MGO-induced insulin dysfunction (reduced secretion and increased resistance) may directly cause vascular dysfunction, which is a common complication of diabetes. In patients with neurodegenerative diseases, the levels of active aldehydes are also increased significantly[10]. The levels of 4-hydroxynonenal and acrolein in the brain of patients with mild cognitive impairment and early Alzheimer's disease are increased, and the neurotoxicity of acrolein is time- and concentration-dependent[11,12]. In addition, MGO can be detected in the arterial wall of a rat model of middle cerebral artery ischemia-reperfusion[13].

In a metabonomic study, GC-ion mobility spectrometry was used to analyze changes in exhaled VOCs in mild cognitive impairment, Alzheimer's disease, and normal control groups. Six compounds (tentatively acetone, 2-propanol, 2-butanone, hexanal, heptanaldehyde, and 1-butanol) play key roles in the diagnosis of mild cognitive impairment and Alzheimer's disease[14]. In addition to the detection of cognitive-related volatile substances, such as 2-propanol and hexanal, our analyses also revealed the metabolic pathways of 2-propanol and MGO (Figure 4). Therefore, the increase in serum 2-propanol levels in pregnant women with GDM may be a potential marker of MGO and cognitive decline.

In the urine analysis, 11 different metabolites were detected. Five of these were highly expressed in the GDM group: Benzene; 1,3-bis(1,1-dimethylethyl)-cyclohexanone; phenol; 1-hexanol, 2-ethyl; and 3,4-dimethylcyclohexanol. The ROC curves of these volatile substances correlated well, with AUCs > 0.5. The AUC for benzene was close to 0.9, and the AUCs for cyclohexanone, 1-hexanol, and phenol exceeded 0.5. Under the action of cytochrome P450 monooxygenase, human benzene is oxidized into toxic epoxy benzene, which combines with glutathione to form phenylmercaptouric acid. The latter is metabolized into phenol, catechol, hydroquinone, and other compounds and finally discharged from the body in the form of a sulfate conjugate or glucosidic acid. Cyclohexanone may be formed by oxidation of cyclohexane. Different amounts of cyclohexanone have been found in the exhaled breath of healthy individuals and patients with chronic obstructive pulmonary disease[15]. The levels of ethylhexyl alcohol in the blood and exhalate are reportedly reduced in patients with thyroid papillary cancer and colorectal cancer, respectively. This may be due to the consumption of ethylhexanol during tumor cell proliferation. Various lung cancer cells release 2-ethyl-1-hexanol[16]. These substances may also be related to the metabolic changes caused by oxidative stress and inflammatory changes in pregnant women with GDM.

An increasing number of metabonomic analyses of urine reflect the potential value of urine as an excreted product that can be collected non-invasively for analysis. Metabonomic methods for urinalysis can yield relatively complete metabonomic profiles and provide a new analytical approach for the diagnosis and mechanistic analysis of diseases. Metabolism of oral hypertension drugs, such as losartan, in patients with T2DM studied by GC has shown that plasma metabolites do not change, whereas urine metabolites (sorbitol and inositol) change significantly[17]. Diaz *et al*[18] analyzed the changes in urine metabolism in pregnant women in early, middle, and late pregnancy and found 21 different metabolites,


A
ÐÞ¸ÄÄò.M1 (PCA-X)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.295851
R2X[2] = 0.221649
Ellipse: Hotelling T2 (0.95)
B
ÐÞ¸ÄÄò.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]
Colored according to Obs ID (group)
GDM
NP
t[2]
t[1]
R2X[1] = 0.282552
R2X[2] = 0.0802674
Ellipse: Hotelling T2
C
ÐÞ¸ÄÄò.M2 (PLS-DA)
t[Comp. 1]/t[Comp. 2]/t[Comp. 3]
Colored according to Obs ID (group)
GDM
NP
D
ÐÞ¸ÄÄò.M2 (PLS-DA): Validate Model
$M2.DA1 Intercepts: R2=(0.0, 0.425), Q2=(0.0, -0.33)
R2
Q2
200 permutations 4 components
E
ROC Curve
Sensitivity
1-specificity
Diagonal segments are produced by ties.
Source of the curve
Benzene, 1,3-bis(1,1-dimethylethyl)-
2-Pentanone
4-Fluoro-2-trifluoromethylbenzoic acid, cyclohexylmethyl ester
Thiophene, 3,3'-(1,2-ethenediyl)bis-
Trisiloxane, 1,1,3,3,5,5-hexamethyl-
Silane, dimethyl (dimethyl(dodec-2-enyloxy)silyloxy) propoxy-
Cyclohexanone
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Phenol
1-Hexanol, 2-ethyl-
Cyclododecanol
3,4-Dimethylcyclohexanol

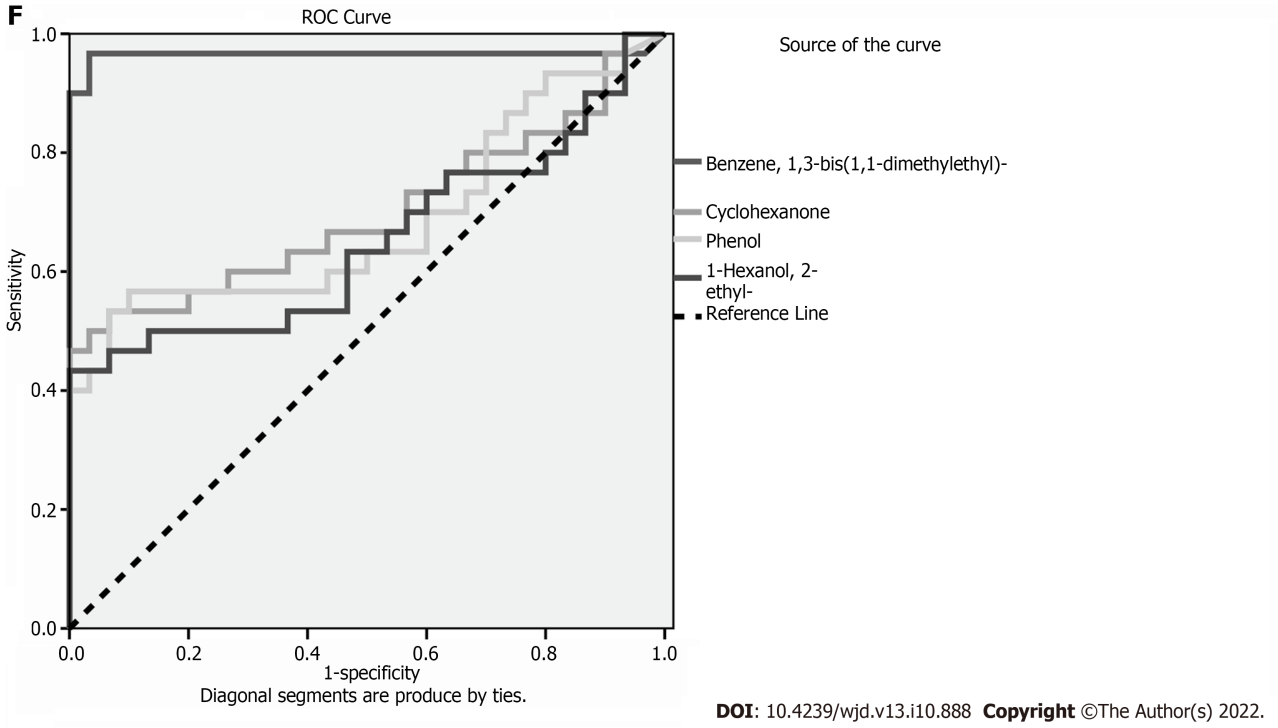

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Figure 3 Metabolomic analysis of urine between groups. A: Principal component analysis score plot; B and C: Partial least squares-discriminant analysis (PLS-DA) score plots ($R^2X[1]$ = 0.295851, $R^2X[2]$ = 0.221649, T^2 = 0.95); D: PLS-DA validation plot intercepts: R^2 = (0.0, 0.425), Q^2 = (0.0, -0.33); E and F: Receiver operating characteristic curves of differential substances from urine. GDM: Gestational diabetes mellitus; NP: Normal pregnancy; ROC: Receiver operating characteristic.

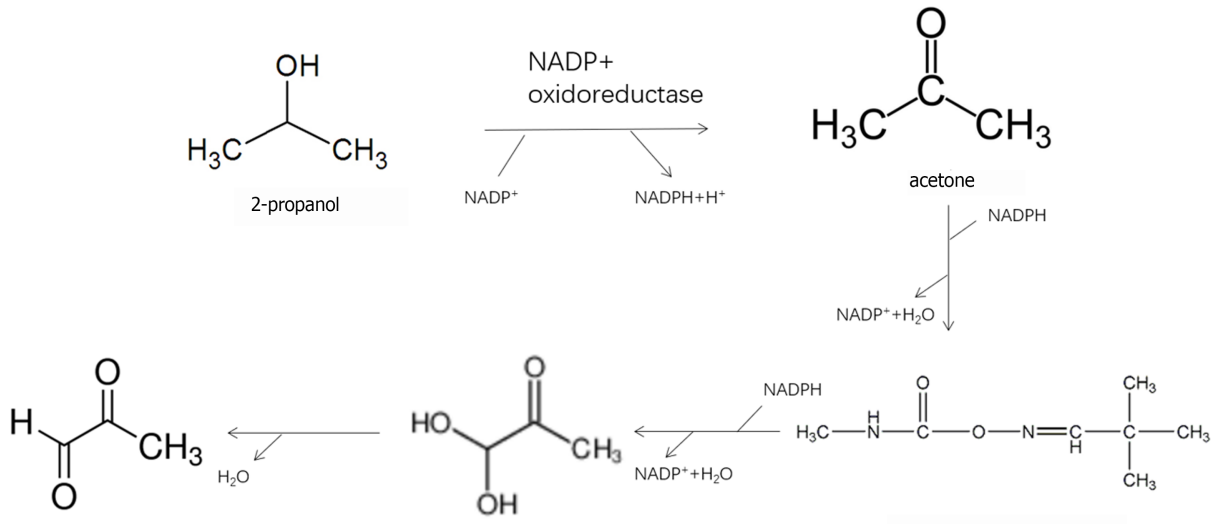

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Figure 4 Pathway of 2-propanol conversion to methylglyoxal.

including choline, creatinine, and lactate[18]. Other metabonomics analyses of the urinary metabolites of GDM pregnant women correlated p-inositol phosphate polysaccharide (P-IPG) with maternal blood glucose and pointed out that P-IPG may be a potential marker of insulin resistance in pregnant women with GDM[19]. Changes in urinary metabolites were observed in GDM patients from 8 to 16 wk postpartum[20]. The authors found that the longer the pregnancy cycle, the higher the lactose content in the urine samples of GDM pregnant women. However, lactose decreased rapidly after termination of pregnancy; in contrast, the blood concentrations of glucose and citric acid increased[20].

In this study, differential volatile metabolites in the serum and urine of pregnant women with GDM were detected by SPME. Volatile substances, such as hexanal, 2-octen-1-ol, and 2-propanol, were found in the serum of pregnant women with GDM. ROC curves indicated that they had a good correlation

with GDM, which may be potential markers. Additional analyses demonstrated the metabolic conversion of 2-propanol in GDM serum to MGO, which could cause systemic damage. Thus, 2-propanol may be a potential marker of MGO. This should be investigated further. Volatile substances, such as benzene, cyclohexanone, 1-hexanol, and phenol, were found in the urine of pregnant women with GDM. However, their metabolic sources require further study.

CONCLUSION

Differential volatile metabolites in the serum of pregnant women with GDM mainly include hexanal, 2-octen-1-ol, and 2-propanol. The differential volatile metabolites in the urine of pregnant women with GDM include benzene, cyclohexanone, 1-hexanol, and phenol.

ARTICLE HIGHLIGHTS

Research background

Gestational diabetes mellitus (GDM) is a metabolic disorder in which hyperglycemia develops during pregnancy in non-diabetic women.

Research motivation

Gas chromatography-mass spectrometry (GC-MS) was used to analyze changes in metabonomics in pregnant women with GDM and to explore the mechanism of cognitive function decline in pregnant women with GDM.

Research objectives

To study the cognitive function of pregnant women with GDM and to identify potential volatile markers to evaluate the cognitive impairment of pregnant women with GDM.

Research methods

Solid-phase microextraction GC-MS analysis was used to detect organic matter in plasma and urine samples. The statistical methods used were principal component analysis and partial least squares-discriminant analysis.

Research results

Differential volatile metabolites in the serum of pregnant women with GDM mainly included hexanal, 2-octen-1-ol, and 2-propanol. The differential volatile metabolites in the urine of pregnant women with GDM included benzene, cyclohexanone, 1-hexanol, and phenol.

Research conclusions

Of 2-propanol may be a potential volatile marker to evaluate the cognitive impairment of pregnant women with GDM.

Research perspectives

The study of perinatal cognitive decline is worthwhile, especially in women with GDM. The key is the prevention and treatment of the disease. Whether 2-propanol can be used as a therapeutic target requires further investigation.

FOOTNOTES

Author contributions: Sana SRGL, Chen GM, Lv Y, Guo L, and Li EY designed the research study; Sana SRGL, Chen GM, Lv Y, and Guo L performed the research, and contributed new reagents and analytic tools; Sana SRGL analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University.

Clinical trial registration statement: This study is registered with the Chinese Clinical Trial Registry (No. ChiCTR2000038703).

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at lienyou_1111@163.com.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

Country/Territory of origin: China

ORCID number: Si-Ri-Gu-Leng Sana 0000-0001-6556-2934; Guangmin Chen 0000-0003-0568-1848; Yang Lv 0000-0002-8878-422X; Lei Guo 0000-0002-9576-2918; En-You Li 0000-0003-0282-8453.

S-Editor: Fan JR
L-Editor: Wang TQ
P-Editor: Cai YX

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 November 15; 13(11): 900-1000

Published by Baishideng Publishing Group Inc

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NAME OF JOURNAL

World Journal of Diabetes

ISSN

ISSN 1948-9358 (online)

LAUNCH DATE

June 15, 2010

FREQUENCY

Monthly

EDITORS-IN-CHIEF

Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS

https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE

November 15, 2022

INSTRUCTIONS TO AUTHORS

https://www.wjgnet.com/bpg/gerinfo/204

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ARTICLE PROCESSING CHARGE

https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS

https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION

https://www.f6publishing.com

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World Journal of Diabetes

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World J Diabetes 2022 November 15; 13(11): 900-911

DOI: 10.4239/wjd.v13.i11.900

ISSN 1948-9358 (online)

REVIEW

Type 2 diabetes and bone fragility in children and adults

Maria Felicia Faienza, Paola Pontrelli, Giacomina Brunetti

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Lee KS, South Korea; Munteanu C, Romania; Zhao CF, China

Received: May 30, 2022
Peer-review started: May 30, 2022
First decision: August 7, 2022
Revised: August 17, 2022
Accepted: October 11, 2022
Article in press: October 11, 2022
Published online: November 15, 2022

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Abstract

Type 2 diabetes (T2D) is a global epidemic disease. The prevalence of T2D in adolescents and young adults is increasing alarmingly. The mechanisms leading to T2D in young people are similar to those in older patients. However, the severity of onset, reduced insulin sensitivity and defective insulin secretion can be different in subjects who develop the disease at a younger age. T2D is associated with different complications, including bone fragility with consequent susceptibility to fractures. The purpose of this systematic review was to describe T2D bone fragility together with all the possible involved pathways. Numerous studies have reported that patients with T2D show preserved, or even increased, bone mineral density compared with controls. This apparent paradox can be explained by the altered bone quality with increased cortical bone porosity and compromised mechanical properties. Furthermore, reduced bone turnover has been described in T2D with reduced markers of bone formation and resorption. These findings prompted different researchers to highlight the mechanisms leading to bone fragility, and numerous critical altered pathways have been identified and studied. In detail, we focused our attention on the role of microvascular disease, advanced glycation end products, the senescence pathway, the Wnt/β-catenin pathway, the osteoprotegerin/receptor-activator of nuclear factor kappa B ligand, osteonectin and fibroblast growth factor 23. The understanding of type 2 myeloid bone fragility is an important issue as it could suggest possible interventions for the prevention of poor bone quality in T2D and/or how to target these pathways when bone disease is clearly evident.

Key Words: Type 2 diabetes; Bone remodeling; Cytokines; Bone fragility; Bone mineral density; Chronic kidney disease

Core Tip: Type 2 diabetes (T2D) patients show increased susceptibility to bone fractures, despite their bone mineral density being normal or increased, leading to difficult identification for clinicians. The prevalence of T2D in adolescents and young adults is increasing alarmingly. Different researchers highlighted the mechanisms leading to bone fragility, and different critical altered pathways have been identified and studied. In this review, we described the different metabolic pathways responsible for bone fragility in patients with T2D. They can be useful for its management, although further studies are needed to deepen our understanding of the mechanisms underlying bone fragility in T2D.

Citation: Faienza MF, Pontrelli P, Brunetti G. Type 2 diabetes and bone fragility in children and adults. *World J Diabetes* 2022; 13(11): 900-911
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/900.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.900

INTRODUCTION

The prevalence of type 2 diabetes (T2D) mellitus in adolescents and young adults is increasing alarmingly. Data from the SEARCH study showed an annual increase of approximately 7% in the incidence of T2D among people aged 10-years-old to 19-years-old in the United States, with increases in all ethnic groups[1]. Increases in children, adolescents and young adults with T2D have been described across most regions of the world[2]. The highest T2D incidence rates in youth have been registered in Canadian First Nations, American Indian and Navajo nation, Australian Aboriginal and Torres Strait Islander and African American populations (31-94/100000 each year), while youths from non-Hispanic Caucasian populations (*i.e.,* United States and Europe) display the lowest incidence rates (0.1-0.8/100000 each year). Studies show the highest prevalence in youth from Mexico and Brazil, indigenous populations in Canada and the United States, together with Black populations in the Americas (160-3300/100000). Conversely, the lowest prevalence was registered in European populations (0.6-2.7/100000)[2].

A recent literature review examined country-specific prevalence and incidence data of youth-onset T2D published between 2008 and 2019[3]. The highest prevalence rates of youth-onset T2D were observed in China (520 cases/100000 people) and the United States (212 cases/100000) and the lowest in Denmark (0.6 cases/100000) and Ireland (1.2 cases/100000). However, the highest incidence rates were reported in Taiwan (63 cases/100000) and the United Kingdom (33.2 cases/100000), with the lowest in Fiji (0.43 cases/100000) and Austria (0.6 cases/100000). These differences in epidemiology data may be partially explained by variations in the diagnostic criteria used within studies, screening recommendations within national guidelines and race/ethnicity within countries.

The main predisposing risk factors for the development of T2D in pediatric age are represented by obesity, family history and sedentary lifestyle[4]. The mechanisms leading to T2D in young people are similar to those in older patients. However, the severity of onset, reduced insulin sensitivity and defective insulin secretion can be different in subjects who develop the disease at a younger age[5]. In particular, the phase of nutrient-induced insulin secretion might be impaired earlier in children and adolescents than in older subjects[6].

The comorbidities associated with T2D in young people include hypertension, cardiovascular disease, kidney impairment and retinopathy. Furthermore, psychosocial problems are often observed[7]. Altered bone quality has been reported in patients with T2D, possible mechanisms for the effect of T2D on bone mineral density (BMD) include the toxic effects of hyperglycemia, which may impair differentiation and proliferation of osteoblasts[8]. In addition, hyperglycemia can increase urine calcium excretion, which inhibits bone formation[8]. Thus, the objective of this review was to describe T2D bone fragility together with all the possible involved pathways.

T2D AND BONE IMPAIRMENT

Most studies have found that patients with T2D have preserved, or even increased, BMD compared with controls but display bone fragility with consequent increased susceptibility to fractures[9-11]. This apparent paradox is due to the altered bone quality in these patients. In detail, the spine trabecular bone score is decreased in patients with T2D, and it is a predictor of fracture risk independently of the BMD [12].

Furthermore, different studies have evaluated T2D effects on bone microarchitecture of the peripheral skeleton (radius and tibia) through high-resolution peripheral quantitative computed tomography. These studies have generally shown preserved, or even improved, trabecular bone microarchitecture in patients with T2D compared with controls[12-17]. Furthermore, some[14,15,17-19], but not all[16,20,21], studies report augmented cortical porosity in patients with T2D, and interestingly this parameter independently predicts fracture risk.

Another aspect of bone quality that might be impaired in patients with T2D is the mechanical characteristics that can be assessed through the measurement of the bone material strength index. However, discordant results have been reported on this issue. In detail, some authors found reduced bone material strength index in T2D in comparison with controls[20,22,23], whereas others reported no significant variation[16]. These different results could be associated to the different comorbidities characterizing T2D.

Several studies have noted reduced bone turnover in patients with T2D[20,24,25]. It has been reported that patients with T2D have reduced markers of bone formation [serum levels of procollagen type 1 amino-terminal propeptide and osteocalcin (OC)] as well as resorption (carboxy-terminal telopeptide of type 1 collagen)[20,24,26,27]. Moreover, Starup-Linde *et al*[28] demonstrated an inverse relationship between glycemic control (hemoglobin A1c) and OC levels and a similar trend for carboxy-terminal telopeptide of type 1 collagen and procollagen type 1 amino-terminal propeptide. N-terminal telopeptide of type 1 collagen and bone-specific alkaline phosphatase levels are not significantly different between patients with T2D and controls[29]. Very recently, it has been reported that thyroid homeostasis could affect bone turnover markers[30] and that follicle-stimulating hormone levels may contribute to the suppression of the same markers[31].

MECHANISMS AND MOLECULAR PATHWAYS INVOLVED IN T2D BONE FRAGILITY

An indication of mutual regulatory control of both bone and glycemic homeostasis recognizes the close interplay between these two systems. The common regulatory mechanisms involve microvascular disease, advanced glycation end products (AGEs), osteoprotegerin (OPG)/receptor-activator of nuclear factor kappa B ligand (RANKL), the Wnt/β-catenin pathway, osteonectin and fibroblast growth factor 23 (FGF23) (Table 1).

Microvascular disease

Microvascular disease is a common complication (retinopathy, nephropathy or neuropathy) of diabetes [32]. Angiopathy has been demonstrated in the iliac crest of diabetic patients[33]. Recently, decreased microvascular blood flow has been demonstrated to be linked with cortical porosity in patients with T2D, suggesting that microvascular disease negatively affects bone microarchitecture in T2D[16]. Consistently, cortical porosity of the distal radius and tibia is most pronounced in patients with T2D with microvascular disease[19]. In contrast, in 2022 it was found that the poorest femoral trabecular microarchitecture was associated with vascular complications in patients with T2D[34]. Patients with T2D with microvascular disease display a significantly lower trabecular bone score, after adjusting for confounders. Moreover, multivariable analysis demonstrated a significant correlation between low 25(OH) vitamin D levels and microvascular disease[35].

Several mechanisms have been proposed to explain how microvascular disease is associated with bone fragility in T2D. It is important to remember that skeletal blood flow provides growth factors, hormones, oxygen and nutrients affecting bone remodeling, suggesting that alteration in microvasculature leads to bone impairment. In the same manner as perivascular cells show stem-cell like properties and may differentiate in osteoblastic cells, blood vessels also release factors affecting the differentiation and activity of osteoblasts and osteoclasts[36]. Blood flow promotes angiogenesis and thus osteogenesis. Bone blood flow is reduced in T2D rats[37], and hypoxia increases the canal network in rat cortical bone [38], suggesting that insufficient oxygen and blood flow associated with microvascular disease alters bone microarchitecture. The recruitment of osteoprogenitors from blood vessels is fundamental for bone formation following osteoclast resorption[39]. Thus, microvascular disease could uncouple resorption and formation in cortical bone by impairing osteoprogenitor recruitment. However, further studies are needed to deepen our understanding of the mechanisms and in particular whether bone fragility is a comorbidity of T2D or a complication (this item is a matter of debate)[40].

AGEs

Hyperglycemia disturbs both bone cells and the extracellular matrix. The presence of glucose determines the production of intermediate products, which eventually generate the irreversible accumulation of AGE[41]. AGE accumulation leads to the synthesis of defective collagens as well as of reactive oxygen species, with consequent structural changes in the bone[42]. In detail, considering the organic bone matrix, these products lead to diminished bone strength[43,44]. Elevated AGE levels are associated with increased fracture risk[45].

Table 1 Mechanisms of bone fragility in type 2 diabetes

Cytokines/factors	Mechanisms	Bone effect
Microvascular disease	Reduced bone vasculature, blood flow and oxygen supply	Increased fracture risk
AGEs	Osteoclast and osteoblast alterations	Poor bone quality, impaired biomechanical properties, and occurrence of fracture
Senescence pathways	Osteocyte impairment	Reduced biomechanical strength, defective bone microarchitecture and increased risk of fracture
Wnt/β-catenin pathway	High levels of sclerostin and DKK1 in T2D. Involvement in CKD-MBD	Impairment of bone cell activity in murine and human models
OPG/RANKL	Decreased OPG/RANKL ratio	Suppressed bone turnover
Osteonectin	High levels of osteonectin	Albuminuria is linked to higher levels of osteonectin
Osteocalcin	Reduced levels in T2D	Decreased bone formation. Bone fracture, involved in T2D and kidney complication
FGF23/klotho	High FGF23 and low klotho levels in T2D	Dysregulation of mineral metabolism, bone fractures. FGF23 is linked to bone fragility; reduced klotho levels are predictors for CKD-MBD

AGEs: Advanced glycation end products; CKD-MBD: Chronic kidney disease-mineral and bone disorder; DKK1: Dickkopf-related protein 1; FGF23: Fibroblast growth factor 23; OPG/RANKL: Osteoprotegerin/receptor-activator of nuclear factor kappa B ligand; T2D: Type 2 diabetes.

The AGE-receptor for AGE (RAGE) binding generates reactive oxygen species production, macrophage and platelet activation, vascular inflammation and inflammatory cell migration[46]. All these events are involved in the onset and progression of typical macro- and microangiopathy associated with diabetes, thus leading to brittle bones with diminished strength and less capability to deform before fracturing[47].

RAGE is also expressed by immune cells and incites activation of the nuclear factor kappa-light-chain-enhancer of activated B cells, a central transcription factor of the immune and inflammatory response[46]. The AGE-RAGE interaction in immune cells leads to the increased expression of chemokines and adhesion molecules, secreting further RAGE ligands, supporting the inflammatory tissue response, regulating the activated macrophage reaction to enhance the destructive signals in the tissues and inhibiting the repair and remodeling responses[46]. AGEs may determine osteoclastogenesis and osteoblast alterations in the bone microenvironment due to the increase in inflammatory cytokines, leading to osteoporosis[48].

In detail, pentosidine, the most studied AGE, accumulates in the trabecular and cortical bone in patients with T2D and negatively affects their bone strength as well as probably leading to functional changes in osteoblasts and the bone mineralization process[49,50]. Consequently, trabecular and cortical bones show impaired biomechanical properties and decreased strength, together with altered osteoblast activity as well as adhesion to the collagen matrix and thus negatively affect bone homeostasis[45,50-52].

AGE bone content correlates with worse bone microarchitecture, including lower volumetric BMD, bone volume/total volume and increased trabecular separation/spacing[53]. High concentrations of AGEs blunt insulin-like growth factor I- mediated osteoblast stimulation and determines the resistance of osteoblasts to insulin-like growth factor I effects[54]. Consistently, insulin-like growth factor I serum levels have been found to be inversely correlated with the occurrence of vertebral fractures in T2D postmenopausal women[55].

The role of cellular senescence in mediating skeletal fragility in T2D

Different forms of stress can lead a cell to enter an irreversible permanent growth arrest known as senescence[56]. This is triggered by cyclin-dependent kinase inhibitors, remarkably p16Ink4a and p21Cip1, that antagonize the activity of cyclin-dependent kinases to stop cell proliferation[57,58]. Senescent cells display a transformed gene expression profile with an increase in senescent cell anti-apoptotic pathways as well as a senescence-associated secretory phenotype[59], typically consisting of proinflammatory cytokines, chemokines and matrix remodeling proteins[60,61]. A premature increase in senescent cells is evident in T2D, especially pancreatic β cells and bone[62,63]. In particular, osteocyte senescence has been demonstrated using an inducible obese mouse model of T2D. These mice display bone quality alterations quite similar to bones from humans with T2D, such as reduced biomechanical strength, defective cortical bone microarchitecture and low bone formation rates[63]. Furthermore, in this model, senescent osteocytes were identified for the high levels of p16Ink4a and p21Cip1, senescence-associated distension of satellites, increased telomere-associated foci (another cell marker of senescence) as well as typical increased expression of proinflammatory senescence-associated secretory phenotype and nuclear factor kappa-light-chain-enhancer of activated B cells[63].

Additionally, cellular senescence in T2D has been linked to the incidence of fracture in murine models and patients[64,65]. In detail, using a murine model of T2D reflecting both hyperinsulinemia caused by insulin resistance induced by a high-fat diet and insulinopenia induced by low dose streptozotocin, increased density of senescent cells has been demonstrated in the callus area in fracture healing [64]. Additionally, the same authors reported that cells of the osteoblastic lineage cultured with sera from patients with T2D displayed increased expression of the p53 responsive genes that are typical of a senescent microenvironment[64]. The decreased levels of serum senescent miR-31-5p in older diabetic women is linked to incidents of fragility fracture and can significantly predict fracture risk if combined with femoral neck and BMD measurements[65].

The Wnt/β-catenin pathway

The Wnt/β-catenin pathway activation promotes osteoblastogenesis and bone formation but inhibits osteoclastogenesis. Dickkopf-related protein 1 and sclerostin (encoded by *Sost*) antagonize the Wnt/β-catenin pathway by binding to low-density lipoprotein receptor-related protein 5 or 6, thus inhibiting osteoblastogenesis and promoting osteoclastogenesis[66].

Bone expression of sclerostin and Dickkopf-related protein 1 has been demonstrated to be high in T2D rat models[67,68]. Circulating sclerostin levels have also been found to be increased in patients with T2D[69] and correlated to the decrease in bone formation markers[70]. In contrast, in T2D postmenopausal women the high circulating levels of sclerostin are related to vertebral fractures[71]. Interestingly, T2D postmenopausal women with previous fractures display thinner cortical bone, together with a tendency towards larger volumetric bone density and elevated circulating levels of sclerostin compared with diabetic women without fractures and nondiabetic controls with fractures [72]. More recently, Piccoli *et al*[53] reported that *Sost* expression in RNA extracts from the femoral head of patients with T2D is significantly increased compared with the controls, although circulating sclerostin levels were found to be higher in T2D subjects but not statistically significant.

OPG/RANKL

OPG is a soluble tumor necrosis factor receptor superfamily member originally discovered in bone[73,74]. It is an anti-resorptive cytokine that works by binding and neutralizing the receptor activator for RANKL. RANKL is a molecule that induces osteoclast differentiation and activity[73,74]. The OPG/RANKL axis is also linked to the regulation of glucose homeostasis[75,76]. In detail, hyperglycemia downregulates RANKL expression, which inhibits the differentiation and activity of osteoclasts[73,76].

The duration of diabetes seems to negatively affect bone metabolism, but poor glycemic control (hemoglobin A1c ≥ 7.5%) has also been shown to be associated with an increased risk of fracture[77]. Decreased levels of RANKL have been reported in diabetic patients compared to healthy subjects[78]. This seems to be due to the increased number of immature osteoblasts and osteoclasts[79]. Other authors have reported that serum RANKL levels are reduced and OPG increased in diabetic patients with respect to nondiabetics and prediabetic subjects[80,81].

Furthermore, it has also been reported that high RANKL levels are related to a significantly increased risk of T2D development[82]. However, other authors did not measure significant differences in RANKL levels between patients with T2D and controls[29]. Human osteoblast cultures from cancellous bone biopsies of diabetic patients displayed a decreased RANKL/OPG ratio compared to the controls, suggesting that the bone turnover process is suppressed[83].

Osteonectin

Osteonectin is produced by osteoblasts and high osteonectin serum levels represent a marker of bone formation[84]. Osteonectin induces osteoblast differentiation, commitment and survival. *In vivo*, osteonectin-knock out and haploinsufficient mice show osteopenia with low bone turnover, a decreased number of osteoblasts as well as a reduced bone formation rate[85,86]. Additionally, Dole *et al*[87] reported that a single nucleotide polymorphism in the 3′ untranslated region of osteonectin determined variability in bone mass by modulating its expression. Patients with albuminuria had significantly higher levels of osteonectin compared with normoalbuminuric patients[88].

T2D AND BONE-KIDNEY CROSS-TALK: THE ROLE OF BONE-DERIVED HORMONES

Chronic kidney disease (CKD) represents a serious complication of T2D and impacts 25%-40% of the diabetic population[89], thus leading to end stage renal disease with the need for dialysis or kidney transplantation[90]. Although kidney replacement therapy improves long-term survival and quality of life in CKD patients, this survival highlights bone fragility as an emerging complication[91]. In a large cohort of patients with CKD followed between 1990 and 1999, Bal *et al*[92] demonstrated that the

fracture risk was higher with a prolonged period of dialysis before transplantation, and both epidermal growth factor receptor decrease and albuminuria increase were considered important risk factors for fracture[93]. Bone fragility in CKD patients is dependent on several risk factors, and literature data demonstrate the impact of age, race (Caucasian) and sex, low body mass index < 23 kg/m^2, glucocorticoid duration and immunosuppressive agents[94]. However, in addition to the described factors and dialysis vintage, diabetes and pancreas replacement therapy are also important risk factors for bone fragility[95,96].

In 2009, the Clinical Practice Guidelines for the Diagnosis, Evaluation, Prevention, and Treatment of CKD-Mineral and Bone Disorder (CKD-MBD) were originally published by Kidney Disease: Improving Global Outcomes[97]. This clinical syndrome defines a systemic disorder in CKD patients responsible for abnormalities in mineral metabolism, bone remodeling and vascular calcification. Despite the completion of several key clinical trials since the 2009 publication of the CKD-MBD guidelines, large gaps in the knowledge still remain[98]. Prospective studies are needed to determine the value of BMD and bone biomarkers as predictors of fractures[99] as well as the impact of different therapeutic approaches on bone fragility, especially in patients with both diabetes and kidney disease. Recent studies have demonstrated that CKD patients with T2D are at increased risk of bone diseases[100], which could involve FGF23.

FGF23

Ribeiro *et al*[101] described how the FGF23/klotho axis is a predictive factor for fractures in patients with T2D with early CKD and demonstrated that α-klotho and FGF23 independently influenced the occurrence of bone fractures. FGF23 is a bone-derived hormone secreted by osteocytes that regulates phosphate and vitamin D metabolism. It acts in the kidney through FGF receptors and klotho, thus preventing renal tubular reabsorption of phosphorus. FGF23 plays an important role in the development of bone and mineral disorders, and many studies over recent years, including patients with CKD and diabetes, have demonstrated that FGF23 levels increase in CKD patients and have an impact on bone disease, cardiovascular disease and all causes of mortality[102]. FGF23 can also induce secondary hyperparathyroidism by increasing the 24-hydroxylation of vitamin D, and these changes are associated with an increased risk of fracture in dialysis[103]. FGF23 levels are also further raised in CKD patients with diabetes who had had a previous fracture[101], thus underlying the association of a history of prior fracture with increased risk of hip fracture, as observed in all dialysis patients[104]. Moreover, FGF23 may also promote insulin secretion and insulin resistance[105], thus influencing the risk of adverse outcomes, especially under CKD conditions[106]. Thus FGF23 could represent a potential biomarker for CKD progression in diabetes[107] and be associated with multiple risk factors [108], including bone fragility.

FGF23 signaling on target tissues is mediated by FGF receptors and klotho, which functions as a coreceptor to increase the binding affinity of FGF23 for FGF receptors. Klotho can also circulate as a secreted protein and a physiologically active hormone. It has been demonstrated that insulin can stimulate the release of klotho by inducing the cleavage of the extracellular domain of klotho by ADAM10 and ADAM17 in the kidney[109]. Cleaved klotho can thus regulate both the phosphorus and calcium metabolism in the kidney and mineral homeostasis in the body through 1-alpha hydroxylase activity as well as parathyroid hormone and FGF23 secretion[110]. Klotho expression is significantly reduced by several kidney injuries such as glomerulonephritis, acute kidney injury, ischemia/reperfusion injury and delayed graft function[111,112], chronic allograft dysfunction[113,114] and renal cell carcinoma[115]. Low klotho levels are also associated with accelerated aging that can promote dysregulated mineral metabolism and osteoporosis. Thus, reduced klotho levels are considered early factors in the development of CKD-MBD[116,117]. Klotho levels are also compromised in patients with early CKD and diabetes[101], while lower levels of klotho seem to be an independent predictive factor for bone fracture[101].

Sclerostin and OC

The presence of diabetes may also increase sclerostin, an osteocyte-specific protein that inhibits bone formation, and higher serum sclerostin levels are associated with increased fracture rates[118]. Thus, sclerostin has been described as an important factor contributing to CKD-MBD[119]. In diabetic patients with CKD, sclerostin levels start to increase in the CKD-G3 stage, while patients in the CKD-G4/5 stages have dramatically increased levels of circulating sclerostin[120].

OC is another bone-derived hormone whose levels reflect the ability of osteoblasts to form bones [121]. OC is directly associated with glucose metabolism and experimental models show that OC can increase insulin production by pancreatic β cells and insulin sensitivity in peripheral tissues[122]. Moreover, insulin receptor signaling increases the production of OC in osteoblasts[123]. OC levels have been recently associated with the risk of incident diabetes and kidney complications, while increased levels have been described in CKD patients[124,125]. In early CKD patients with diabetes, OC levels independently influence the occurrence of bone fracture[101]. However, further studies are needed to confirm the specific role of OC in the context of diabetes and CKD.

Further research is also needed to assess the diagnostic and prognostic value of these bone turnover biomarkers in the field of CKD-MBD in the context of diabetes. However, the described hormones represent important factors for the development of bone diseases in the context of CKD and may be considered as targets for future clinical trials.

CONCLUSION

The studies reported in the present review describe altered bone quality and the possible mechanisms underlying its pathophysiology. Patients with T2D frequently display bone fragility, which is often an underdiagnosed condition in these subjects. The understanding of its pathophysiology is an important issue as it could suggest possible interventions for the prevention of poor bone quality in T2D. Additionally, the discovery of its pathophysiology could help to target these pathways when bone disease is clearly evident. Thus, the simultaneous use of anti-diabetic drugs and bone treating agents could help to ameliorate the quality of life of patients with T2D. This issue is of particular interest considering the life extension observed. Nevertheless, the possible interventions to improve bone quality in T2D require further investigation, which could determine different treatment approaches through personalized medicine.

FOOTNOTES

Author contributions: Faienza MF wrote the clinical implications; Pontrelli P explored the bone-kidney axis; Brunetti G performed the majority of the writing and coordinated the writing of the paper.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: Filipodia
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 912-920

DOI: 10.4239/wjd.v13.i11.912

ISSN 1948-9358 (online)

MINIREVIEWS

Orthotic approach to prevention and management of diabetic foot: A narrative review

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Miranda C, Italy; Ran X, China

Received: July 15, 2022
Peer-review started: July 15, 2022
First decision: August 7, 2022
Revised: August 24, 2022
Accepted: October 11, 2022
Article in press: October 11, 2022
Published online: November 15, 2022

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Abstract

Diabetic foot is a common complication affecting more than one-fifth of patients with diabetes. If not treated in time, it may lead to diabetic foot ulcers or Charcot arthropathy. For the management of diabetic foot, shoe modifications and orthoses can be used to reduce pressure on the affected foot or provide the foot with increased stability. In addition, the shoe modifications and orthotic devices can relieve patient discomfort during walking. Appropriate shoe modifications include changing the insole material, modifying the heel height, adding a steel shank or rocker sole, and using in-depth shoes. Alternatively, a walking brace or ankle-foot orthosis can be used to reduce the pressure on the affected foot. The purpose of this narrative review was to provide a reference guide to support clinicians in prescribing shoe modifications and foot orthoses to treat diabetic foot ulcers and Charcot arthropathy.

Key Words: Diabetic foot; Foot ulcers; Charcot arthropathy; Shoes; Foot orthosis

Core Tip: Footwear modifications and orthosis in diabetic foot management are aimed to prevent ulcers, protect the foot from external stimuli, and regulate the pressure on the foot. Types of shoe modifications include using an in-depth shoe, combination of insole materials, lifting the heel, applying a rocker sole, and applying an extended steel shank or flare or stabilizer. Orthosis includes prefabricated removable walking brace (such as control ankle motion walker, pneumatic walker, and conformer walking boot), Arizona ankle-foot orthosis, patellar-tendon-bearing orthosis, and Charcot restraint orthotic walker.

Citation: Chang MC, Choo YJ, Park IS, Park MW, Kim DH. Orthotic approach to prevention and management of diabetic foot: A narrative review. *World J Diabetes* 2022; 13(11): 912-920
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/912.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.912

INTRODUCTION

Diabetic foot is an infection, ulceration, or destruction of the tissues of the foot. It is associated with neuropathy and/or peripheral artery disease in the lower extremity of a person with diabetes mellitus [1]. The global annual incidence of diabetic foot ulcers in patients with diabetes was reported to be approximately 2%-5%, with a lifetime risk ranging from 15%-20%[2]. According to a report by the American Diabetes Association, 20% of patients with diabetes are hospitalized because of foot problems [3]. The main underlying causes of diabetic foot ulcer are peripheral neuropathy and ischemia due to peripheral vascular disease[4,5]. Once a foot ulcer develops, it may progress to foot infection, which if incurable, could eventually require limb amputation[4]. Most diabetic foot ulcers are caused by repetitive trauma due to weight-bearing or poorly fitting footwear, and ulcers often recur if the weight-bearing is not suppressed and the biomechanical abnormality of the foot is not controlled[5,6]. Prevention is important for effective management of the diabetic foot, with methods including continuous education and proper footwear for the patients with diabetes[6]. Such methods have been reported to prevent approximately 80% of all limb amputations due to diabetes[7]. Unfortunately, however, many patients are unaware of the diabetic foot, have never received proper education on its prevention, for example by wearing adequate shoes, or often receive treatment only after the problems, such as foot ulcers and neuropathic arthropathy, occur[8]. Therefore, it is essential for clinicians dealing with diabetic foot to be aware of the possible feet abnormalities due to diabetic complications and the importance of educating the patients with diabetes, including wearing the right footwear.

Another disease that can occur frequently as a complication of diabetes is Charcot arthropathy. The pathogenesis of Charcot arthropathy is not clearly known yet. It is believed to be a phenomenon in which the bone is weakened due to severe bone deficiency caused by autonomic nervous system abnormality or a local increase in blood flow to the bone[9]. Weakened bones are prone to fractures even under a very small weight, and the ligaments may also weaken, resulting in dislocation or subluxation [10]. Patients with Charcot arthropathy fail to sense pain in their limbs and continue to apply their weight, leading to bone deformity that further changes the normal force transmission path; this increases the chance of a fracture or dislocation and, consequently, deforms the weight-bearing part [11]. Over time, the process stops, and the deformity becomes permanent. Owing to the dislocation, the load cannot be distributed effectively and stably during walking, resulting in movement limitations, and the persisting bone protrusion eventually causes foot ulcers[12]. Furthermore, Charcot foot is characterized by a collapsed arch[9,10]. The non-surgical treatment for Charcot arthropathy is based on off-loading and edema control principles. The goal is to prevent the foot ulcers by minimizing mechanical stress, reducing edema, and creating structural stability, all of which help in restricting the weight-bearing and stabilizing the joint until the Charcot arthropathy is sufficiently controlled[5].

Orthoses play a very important role in treating diabetic feet that have already developed foot ulcers and Charcot arthropathy. Orthotic devices not only provide stability, limit the joint movement, and control foot deformity, but also relieve the load and evenly distribute the pressure on the foot. As a result, orthoses can effectively heal foot ulcers and control the symptoms of Charcot arthropathy. This review describes the management of diabetic feet using properly fitting shoes and orthoses.

FOOT ULCER CLASSIFICATION

Wagner classified foot ulcers from Category 0-3 based on the loss of protective sensation, foot deformity, and history of ulcer or ischemia[13]. Category 0 applies to cases where none of the following applies: Loss of protective sensation, deformity, callus, weakness, or history of ulceration or ischemia. Such cases are dealt with educating the patients on basic foot care and recommending conventional footwear. Category 1 solely involves the loss of protective sensation, and the use of in-depth shoes or

sneakers, non-molded soft inlays, and total contact orthoses is recommended. Category 2 involves foot deformity along with the loss of protective sensation and requires the use of in-depth shoes or sneakers, custom-molded foot orthoses, and external shoe modifications, if necessary. Category 3 involves all three factors, namely loss of protective sensation, foot deformity, and history of ulcer or ischemia, and requires custom-fabricated, pressure-dissipating accommodative foot orthoses, with additional recommendations for inlay-depth, soft-leather, adjustable-lacing shoes, and external shoe modifications, if necessary[13]. As illustrated above, the number of requirements for orthoses or properly fitting footwear and the complexity of prescriptions increase with the rising risk of foot ulcers.

ADAPTED FOOTWEAR

Ill-fitting footwear is a common cause of foot ulcers, whereas therapeutic footwear plays an important role in reducing the likelihood of foot ulcers[14]. Foot ulcers recur in about half the patients who wear shoes without modifications, compared to a recurrence rate of approximately 20% when an appropriate protective footwear is worn[5]. While prescribing adapted footwear for patients with diabetes, the goals are to protect the feet from the external environment, relieve excessive pressure, reduce impact and shear force, control foot deformity, and stabilize the movement[14]. The primary role of adapted footwear is to protect the feet from further harm by reducing the pressure on the affected area rather than treating the foot ulcers themselves and preventing their occurrence or recurrence[14,15]. The goal should be to reduce the pressure by at least 30% or to less than 200 kPa, where the pressure on the sole is the highest[16].

GENERAL PRINCIPLES FOR PRESCRIBING SHOES FOR PATIENTS WITH DIABETES

The shoe should conform to the shape of the foot, and there should be enough space inside, similar to an in-depth shoe. To determine the appropriate shoe size, the overall foot length, arch length, and foot width should be measured[17]. The shoes must be manufactured to accommodate the first and fifth metatarsophalangeal joints, which represent the widest part of the foot, while the shoe length should be such that there is a space of 1.3-1.6 cm between the longest toe and the tip of the shoe[17]. Additionally, the ball of the shoe should match in width with the ball of the foot, and the counter should not press the starting point of the Achilles tendon[8,17]. The insole should be removable and of triple-depth; leather insole is not recommended, as the primary aim is to minimize shear and friction[8]. The heel of the shoe is manufactured to be typically 2.5-5 cm high. If the height of the heel is over 5 cm, the pressure on the forefoot increases excessively[8,18]. Regarding the plantar surface, especially when the forefoot has an ulcer, a rigid rocker or a rigid rocker sole can reduce the pressure and help in healing of the ulcer[8]. It is recommended to purchase the shoes in the afternoon when the feet are swollen. In addition to checking the shoe size, the shoes must be tried on to check if it can correctly support the shape and the size of the feet when weight load is applied[8,17].

IN-DEPTH SHOE

In-depth shoes are usually blucher-type Oxford shoes that are 0.6-1.3 cm deeper than the conventional shoes. This extra space helps when using insoles and foot orthoses, which are necessary in cases of foot deformity due to Charcot arthropathy[8,17]. The in-depth shoes should have a light weight, good shock absorption capacity, and strong heel counter[8]. In the past, the upper material was mostly made of soft leather, but nowadays, breathable synthetic material is commonly used[8]. In addition, the relatively softer insoles are layered, and the lower the density of the insole, the more is the cushioning at the interface between the foot and the insole[19]. The insole is generally thermoformed to contour the patient's foot, and the outer sole is also modified to further reduce the pressure[19]. In addition to the Oxford shoes, sneakers may also be used. Sneakers have several advantages in terms of the depth, removable insoles, variety of ball widths offered by manufacturers, and diversity of design compared to that of the traditional Oxford shoes, allowing the patient to choose a model according to their personal preference[20]. When purchasing a ready-made footwear, the shoes should be modified if the foot deformity is severe. If the shoes cannot be modified, they must be custom-made[8].

SHOE INSOLE

Well-made custom insoles are necessary to properly distribute the pressure around the foot deformity. One of the ways is to increase the ambient pressure in order to relieve the pressure on a certain part of

the foot; however, this approach tends to be inaccurate and can result in damage to the foot because of an increase in the partial pressure[8]. The insole serves as the backbone of the shoe and secures the upper part to the sole[17]. The insole is manufactured in an accommodative form and divided into a soft, semi-rigid, or rigid material. Soft materials include cross-linked polyethylene foam, open-cell polyurethane foam, sponge rubber, and closed-cell expanded rubber. Although these soft materials are excellent for pressure distribution, they wear out quickly and have poor durability[21]. Semi-rigid materials include firm cross-linked polyethylene foam, ethylene vinyl acetate, and cork composite, which have a longer lifespan than the soft materials. They also function as a support in addition to providing shock absorption and cushioning. Semi-rigid materials are fabricated as custom insoles with three or more layers and typically used together with soft materials to provide a combination of support and compliance[22]. Soft and moldable polyethylene foam is used in the area beneath the plantar surface. Urethane polymer is used in the middle layer to prevent wear and absorb shock. Rigid ethylene vinyl acetate or cork is used as the bottommost layer for support and control of movement[8]. Insoles with rigid materials are made of thermoplastics, acrylics, and carbon fiber composites. Although they are highly durable and offer ample support and control, the rigid materials are difficult to modify and have much lesser shock absorption capacity and offer reduced cushioning and protection. In general, the use of rigid materials is contraindicated for patients with diabetes and neuropathy or a history of foot ulcers[23]. Compression paper or leather is commonly used to make insoles for patients with diabetes. The high-strength compression paper insoles are light in weight and inexpensive. Leather insoles are highly durable and adapt well to the plantar surface, absorb moisture, and provide excellent ventilation. However, the leather insoles are not always used owing to their relatively higher price and heavier weight compared to those of compression paper[17].

EXTERNAL SHOE MODIFICATIONS

Rocker sole

Rocker soles are an effective modification method for changing the plantar pressure and improving gait [17,24]. Rocker soles help to transition smoothly from heel-strike to toe-off without bending the shoe or foot. From the biomechanical perspective, this improves the overall gait by restoring the movement of the foot or ankle joint that was lost due to foot pain or deformation. This also relieves the pressure in a specific area of the plantar surface[8]. The apex of the rocker sole should be located proximal to the area where the pressure should be relieved, and the front end of the rocker sole should be arched from the proximal part of the metatarsal head to the distal end of the outsole. If there is an angle in the rocker sole instead of an arch, the gait will not be smooth[17]. Several types of rocker soles are available, all of which require appropriate modifications to meet the needs of the user. For example, the double rocker sole is a soft rocker sole without an outsole in the midfoot region. The forefoot rocker sole has a rocker angle only in the toe area. The heel-to-toe rocker sole has a rocker angle on both the heels and toes[16, 17].

Solid ankle cushion heel

The shoe heel provides stability to the foot heel and distributes the force applied on the foot to the entire sole[17]. The typical height of the shoe heel is 1.5-2 cm for men and 2.5-3 cm for women, but this could be modified according to the user's needs[25]. Most shoe heels are made up of rigid materials, but, if needed, a flexible heel may also be used to allow some plantar flexion[18]. A typical example is the solid ankle cushion heel (SACH), which has a wedge-shaped shock-absorbing material inserted into the heel. The SACH acts as a buffer during heel contact and mechanically increases the heel traction during the gait cycle, creating a smooth transition from heel-strike to toe-off[26]. Typically, the angle of the SACH is within 30°[17]. The SACH is indicated for patients with ankle or hindfoot stiffness due to metatarsal ulcers or Charcot deformity. It is also used in cases of degenerative arthritis or ankle fixation[17,26].

Extended steel shank

The extended steel shank, made of spring steel or carbon graphite, is located between the layers of the sole from the heel to the toes and serves to reinforce the midfoot region of the shoe[27]. The extended steel shank is typically used in combination with rocker soles to help improve their performance. In addition, the shank prevents the bending of the shoe, restricts the toe and midfoot movement, and further reinforces the driving force after a toe-off during gait[8,17]. However, since the extended steel shank can be easily bent owing to the properties of the material, thermoforming may also be combined for increased rigidity[17].

Flare and stabilizer

The flare and stabilizer modify the inside or outside of the shoe to stabilize the foot and serve as a support for the shoe[28,29]. A flare, typically made of ethylene vinyl acetate, is a structure added to the heel and sole of a shoe to widen the support surface of the shoe[17]. The flare, when used in combination with the rocker sole, also serves to increase the stability while walking[8]. A stabilizer is an

extension made of hardened resin or crepe that is added to the side of the sole to provide greater stability than flares do. A stabilizer is used for patients with severe instability on the medial or lateral side of the hindfoot or midfoot[30].

ORTHOSES

The International Working Group on Diabetic Foot (IWGDF) has published the off-loading guidelines for the appropriate treatment of diabetic foot ulcers[31]. According to this guideline, for people with diabetic ulcers, a removable knee-high off-loading device with an adequate foot-device interface should be selected as the first-choice treatment. Furthermore, a total contact cast (TCC) or a nonremovable knee-high walker is recommended depending on the patient's preference or the level of foot deformity. If nonremovable knee-high off-loading devices are contraindicated, a removable knee-high or ankle-high off-loading device is recommended[31].

Previous studies have reported that using a knee-high off-loading device is a faster treatment approach for foot ulcers than using other off-loading devices[32,33]. However, in actual clinical practice, knee-high off-loading devices are not commonly used, because they are contraindicated in at least half of the patients with diabetic foot ulcers with ischemia or infection[34]. Furthermore, many clinicians consider knee-high off-loading devices to be less effective than other types of devices[34]. However, according to the IWGDF guideline, since knee-high off-loading devices are contraindicated only in severe ischemia or infection, they can be used for mild or moderate ischemia or infection[31]. Additionally, TCC has traditionally been considered a gold standard off-loading treatment option, and knee-high off-loading devices are the first suggested option for patients with diabetic foot. Nevertheless, TCC and knee-high off-loading devices are under-used due to the perception that they are time-consuming to make and not cost-effective. However, since instant TCC and removable cast walkers are commonly used nowadays, it is necessary to improve the clinicians' expertise and awareness of orthoses that can be used in diabetic foot treatment[34-36]. Lastly, some barriers may be related to the patients themselves. The patients often perform weight-bearing activities at home that can strain their feet, as they misunderstand that off-loading treatment should be followed outside the home only[34,37]. Moreover, patients who fail to perceive the seriousness of diabetic foot ulcers are less motivated to use the off-loading devices[37,38]. These challenges can be addressed by educating the patients on their condition and the importance of the off-loading devices[35]. The poor mobility and stability that occur when patients use knee-high off-loading devices can also be problematic. The use of the device may result in a difference in length between the legs making it difficult to walk. For such cases, the shoe height, instead of the device, can be modified or mobility aids, such as a frame, can be used simultaneously[34]. The following paragraphs describe the off-loading devices available for diabetic foot ulcers and Charcot arthropathy.

NONREMOVABLE OFF-LOADING DEVICE

A typical example of a nonremovable walker is the TCC. The TCC is considered the standard treatment for managing neuropathic plantar ulcers and is also used to protect the foot in the early stages of vulnerability to Charcot fracture-dislocation[31,39]. The TCC relieves the pressure and load on the foot by distributing the weight across the entire sole and can prevent the risk of injury to bony prominences, such as the malleolus and tibia[40]. However, its limitations include the requirement of a skilled cast technician to apply the TCC appropriately, high manufacturing costs, and time-consuming process[40]. Moreover, the TCC is contraindicated in the very elderly and patients with infection or severe ischemia, visual or balance problems, varicose veins, or contralateral foot ulcers[40].

PREFABRICATED REMOVABLE WALKING BRACE

A prefabricated removable walking brace is used to treat diabetic feet with ulcers and Charcot arthropathy. The types of walking braces include the boot-type control ankle motion walker that controls ankle movement and uses an arch filler[41,42] (Figure 1A), the pneumatic walker that applies pneumatic pressure to reduce edema and prevent callus formation[43] (Figure 1B), and the conformer walking boot that consists of a molded inner liner that wraps around the foot and leg[44]. All walking braces consist of rigid rocker soles and a protective insole made up of materials such as Plastazote® foams, propylene terephthalate, and Spenco®. The walking braces are designed to immobilize all joints of the ankle and foot as would a TCC[8]. The general advantage of walking braces is that they are easy to wear, making it possible to manage the affected area at a relatively lower cost. However, if the foot deformity is severe, applying them can be difficult, and their large volume may reduce patient compliance[8].

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Figure 1 Types of refabricated removable walking brace and general appearance of orthosis. A: Control ankle motion walker; B: Pneumatic walker; C: Arizona ankle-foot orthosis; D: Patella-tendon-bearing orthosis.

ARIZONA ANKLE-FOOT ORTHOSIS

The Arizona ankle-foot orthosis (AFO) is mainly used for non-surgical management of posterior tibial tendon dysfunction or spastic deformity[45] (Figure 1C). It is also used in cases of ankle instability, arthritis, Charcot arthropathy, and diabetic peripheral neuropathy[8]. The Arizona AFO, typically custom-made from leather and polypropylene, extends proximal to the mid-axis of the tibia and distal to the metatarsal heads. The Arizona AFO plays a role in minimizing valgus alignment of the hindfoot, lateral calcaneal displacement, and dislocation of the medial ankle or restoring inadequate kinematics by supporting the calf and midfoot[46,47].

PATELLAR-TENDON-BEARING ORTHOSIS

The patellar-tendon-bearing (PTB) orthosis is used for partial weight-bearing of the lower extremities while restricting ankle movement[48] (Figure 1D). It supports the patellar tendon so that 60%-70% of the weight is supported by the knee joint and approximately 30% by the ankle joint[18]. A PTB orthosis can be applied to diabetic feet with fractures of the tibia or fibula, foot ulcers, and Charcot arthropathy[17]. The PTB orthosis is custom-made using a plastic-type brim based on the same principle as that used for making a PTB socket for below-knee prosthesis. The load is distributed to the ankle joint of the orthosis through the medial and lateral uprights. In general, the PTB orthosis is designed to restrict the movement of the ankle joint and provide internal and external stability[18]. Although this orthosis has the advantage of easily responding to changes in foot circumference caused by severe fluctuations in lower-extremity edemas, its heavy weight may limit patient compliance[8].

CHARCOT RESTRAINT ORTHOTIC WALKER

The Charcot restraint orthotic walker (CROW) is an orthosis manufactured to completely cover the feet and legs and can be applied to diabetic feet with Charcot arthropathy[8]. In Charcot arthropathy, it is used to prevent the development of ulcers by evenly distributing pressure to the entire leg and foot[49]. For a severe rocker sole deformity in the midfoot, it may be necessary to limit or eliminate the movement of the midfoot and hindfoot to restrict the entire foot and ankle joints. In this case, the CROW eliminates the movement of the foot and ankle joints, reduces the load and shear force applied to the foot, and protects the deformed foot from further damage[50]. The CROW orthosis is similar to a bivalved TCC and consists of a rigid polypropylene anterior and posterior shell with a dorsiflexion stop, heel lift, and rocker sole to counteract the nutcracker effect[8]. Since the CROW orthosis is fastened with a Velcro® strap, it is easy to detach, making it convenient for wound management, including wound cleaning[49]. However, as the CROW orthosis is bulky, it potentially limits patient compliance and sometimes leads to knee and lumbar pain due to immobility of the lower extremities[51].

CONCLUSION

In patients with diabetes, a foot ulcer or Charcot arthropathy is often the first step to amputation of the lower extremity, which becomes a major obstacle in the patient's life. To prevent foot ulcers, thorough and repeated patient education on diabetic feet is necessary in addition to preventive skin care and, above all, prescription of appropriate footwear. Once foot ulcers and Charcot arthropathy occur, it is extremely important to prescribe appropriate footwear and orthoses for the diabetic feet to effectively treat the foot lesion and prevent further deterioration. We believe that this review can serve as a reference guide for medical staff when prescribing appropriate footwear and orthoses for patients with diabetes.

FOOTNOTES

Author contributions: Chang MC and Choo YJ consulted the literature and wrote the first draft, revised, and improved the manuscript; Chang MC, Choo YJ, Park IS, Park MW and Kim DH designed, checked, modified, and finalized the manuscript; and all authors have proofread the revised manuscript.

Supported by the National Research Foundation of Korea Grant that was funded by the Korean Government, No. NRF-2019M3E5D1A02069399.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: South Korea

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 921-925

DOI: 10.4239/wjd.v13.i11.921

ISSN 1948-9358 (online)

MINIREVIEWS

Effects of Chios mastic gum on cardiometabolic risk factors

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kosmas C, United States; Yang L, China

Received: July 24, 2022
Peer-review started: July 24, 2022
First decision: September 12, 2022
Revised: September 16, 2022
Accepted: October 11, 2022
Article in press: October 11, 2022
Published online: November 15, 2022

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Abstract

Chios mastic gum (CMG), the resin produced by the trunk of *Pistachia lentiscus* var Chia, has been used for culinary and medicinal purposes since antiquity. Despite the fact that *Pistacia* species are widely distributed throughout the Mediterranean basin and in the circum-Mediterranean regions, CMG is a distinctive resin of the mastic trees grown exclusively in the southern part of the island of Chios. CMG has been used for centuries as a spice, a cosmetic, but its most important usage has been as a strong phytotherapeutic therapy, primarily for the management of gastrointestinal diseases. Recently, there are studies demonstrating that CMG has hypolipidemic, cardioprotective and antidiabetic properties. Therefore, the aim of the present review is to summarize the existing literature data regarding the potential beneficial effects of CMG on cardiometabolic risk factors.

Key Words: Chios mastic gum; Glucose; Cardioprotection; Low-density lipoprotein-cholesterol; Triglycerides

Core tip: Chios mastic gum (CMG), the resin produced by the trunk of *Pistachia lentiscus* var Chia, has been used for centuries as a strong phytotherapeutic therapy, primarily for the management of gastrointestinal diseases. Recently, there are studies demonstrating that it has hypolipidemic, cardioprotective and antidiabetic properties. Therefore, the aim of the present review is to summarize the existing literature data regarding the potential beneficial effects of CMG on cardiometabolic risk factors.

Citation: Papazafiropoulou AK. Effects of Chios mastic gum on cardiometabolic risk factors. *World J Diabetes* 2022; 13(11): 921-925
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/921.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.921

INTRODUCTION

The aromatic resin known as Chios mastic gum (CMG) is made by the evergreen plant *Pistacia lentiscus* var Chia (Anacardiaceae). Mastic is traditionally produced by making shallow slits in the bark and trunk of the shrub using specific implements called ceditria[1]. Despite the fact that *Pistacia* species are widely distributed throughout the Mediterranean basin and in the circum-Mediterranean regions, CMG is a distinctive resin of the mastic trees grown exclusively in the southern part of the island of Chios, which is situated in the central Aegean Sea close to the coastline of Minor Asia. The fact that mastic is only produced in one location of the island and nowhere else in the greater Mediterranean region may be explained by thousands of years of selective cultivation and the particular microenvironment. The mastic tree's cultivation and resin harvesting are part of the area's cultural heritage, and the total production comes from 24 settlements (Mastichochoria in Greek)[1].

CMG has been used for centuries as a spice, a cosmetic, but its most important usage has been as a strong phytotherapeutic therapy, primarily for the management of gastrointestinal diseases. Galenos and Dioscourides, two ancient Greek physicians, highlighted its benefits and suggested using it. Furthermore, the need for CMG has always held a special place in folk medicine throughout Europe and Asia during the Byzantine and Medieval eras, and afterwards in formal Pharmacopeias[2]. The first research revealing the resin's positive characteristics on gastrointestinal inflammations, and particularly those caused by *Helicobacter pylori*, were published in the 1980s, reigniting the scientific community's interest in CMG[3].

The most prevalent and traditional therapeutic application of mastic in the treatment of gastrointestinal diseases has been extensively studied in recent decades by several scientific investigations that have focused on CMG. Its antibacterial, anti-inflammatory, antioxidant, hypolipidemic, antidiabetic, and anticancer activities have since been the subject of several investigations[2]. Therefore, the aim of the present review is to summarize the existing literature data regarding the potential beneficial effects of CMG on cardiometabolic risk factors.

CHEMICAL COMPOSITION OF CMG

Numerous chemicals have been isolated and identified after a detailed analysis of the chemical makeup of CMG[8-12]. However, ongoing study continues to uncover novel substances, as seen in the case of mastichinoic acid A, a new tetracyclic triterpenoid that was recently discovered from CMG[13]. About 25% of the total CMG is made up of poly-b-myrcene, a sticky and insoluble polymer. From CMG, a number of triterpenoids have been identified. More specifically, acidic and neutral fractions can be obtained from complete mastic gum extract (without the polymer). All significant triterpenic acids, including masticadienonic, isomasticadienonic, oleanonic acid, moronic acid, masticadienolic acid, and oleanolic acid, are included in the acidic fraction. Triterpenic neutral substances such as oleanolic aldehyde, 28-norolean-17-en-3-one, tirucallol, b-amyrone, isomasticadienolic aldehyde, and dammaradienone are included in the neutral fraction.

Other substances with smaller amounts include verbenone, a-terpinolene, and linalool, which support the antibacterial properties of mastic oil, and camphene, which has hypolipidemic properties [14]. Gallic acid traces have also been found. It is amazing that research describing the antibacterial, hypolipidemic, and anti-inflammatory properties of mastic gum or mastic oil have shown the presence of synergy phenomenon, where the combination of many substances is more potent than any one ingredient alone. With herbal products that include numerous different active ingredients, this synergy phenomenon occurs frequently.

EFFECTS OF CMG ON LIPIDS METABOLISM

Human low-density lipoprotein cholesterol (LDL-C) has been shown to be resistant to copper-induced oxidation *in vitro* through the powerful antioxidant effects of CMG[15]. Peripheral blood mononuclear cells are cytotoxic when exposed to oxidized LDL-C without the presence of CMG, an d whole polar extract of CMG prevents this from happening. While mastic complete polar extract increases glutathione (GSH) levels and lowers CD36 expression, oxidized LDL-C decreases GSH levels and increases CD36 expression[16]. Rats susceptible to detergent-induced hyperlipidemia and naïve rats have both been

used to study the hypolipidemic effects of mastic gum essential oil (MGO). The levels of blood total cholesterol, LDL-C, and triglycerides were decreased in a dose-dependent manner after MGO treatment to untrained rats. MGO injection resulted in a significant decrease in the levels of total cholesterol, LDL-C, and triglycerides in hyperlipidemic rats[16].

In a different study, complete CMG was given as a powder and blended with food for 8 wk in low and high doses to examine the hypolipidemic effects of CMG on diabetic mice. The serum levels of triglycerides, total cholesterol, and LDL-C were all significantly lower in the low-dose group whereas high-density lipoprotein cholesterol (HDL-C) levels were significantly higher. Triglyceride levels were considerably lower in the high-dose group[17].

When administered to hypercholesterolemic rabbits, complete mastic extract without polymer and neutral mastic fraction (NMF) decreased total cholesterol levels by 47% and 88%, respectively, exhibiting strong hypolipidemic actions[18]. Healthy adults over the age of 50 years have received total mastic extract. Subjects were divided into two groups at random and given either a mastic solution (low dose) for 12 mo or a daily dose of 5 g of mastic powder (high dose) for 18 mo. The high-dose group showed a decrease in blood total cholesterol, LDL-C, total cholesterol/HDL-C ratio, apolipoprotein A-1, and apolipoprotein B, but no change in the apoB/apoA-1 ratio[19].

In a prospective, randomized, placebo-controlled, pilot study, healthy volunteers' total cholesterol and blood sugar levels were considerably reduced over the course of 8 wk by taking three capsules per day containing 330 mg CMG. It is important to note that, despite the absence of side effects, overweight and obese people in particular shown excellent tolerance. CMG activity decreases when polymer is absent. Measurements of cholesterol levels in healthy individuals did not reveal any appreciable reduction after taking mastic gum capsules free of polymers[20].

A major limitation of the above human studies was that the rest of the diet of the participants (apart from the addition of CMG) was not controlled and, therefore, any effect of possible diet changes on the results of the study could not be excluded. It should be mentioned that the effects of CMG on lipids in humans are rather minor and should not be overstated as unique, as there are other natural substances, such as sterols and stanols, that have been shown to cause significant reductions in LDL-C.

EFFECTS OF CMG ON CARDIOPROTECTIVE ACTIVITY

Cardiovascular disease risk appears to be decreased by CMG. Perhaps one of the underlying causes of this function is the potent antioxidant activity of CMG and its ability to inhibit the buildup of the oxidized LDL in cells, which can cause atherosclerosis[16]. Two essential adhesion molecules can be decreased by the neutral fraction of CMG (25–200 g/mL) and, more specifically, the chemical tirucallol (0.1–100 mmol/L), according to research in human aortic endothelial cells [vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1]. Due to the buildup of monocytes in the artery innermost layer, VCAM-1 and ICAM-1 are linked to the early development of atherosclerosis [21]. In another study, male 12-wk-old diabetic mice were divided into groups receiving low and high doses of CMG. The high-dose CMG group (n = 12) received 500 mg/kg body weight for the same duration as the low dose CMG group (n = 12) for a total of 8 wk. CMG lowered serum lipid and glucose levels in both groups[22]. In 2018, the authors showed that administering CMG to renovascular hypertensive rats at a dose of 40 mg/kg/d for 2 wk after the onset of hypertension lowered their blood pressure. The results showed a relationship between reduced levels of renin, C-reactive protein, and interleukin-6, as well as increased vascular and cardiac remodeling[23].

In a different *in vivo* experiment, for 6 wk, rabbits were fed a specific diet supplemented with the NMF and the total mastic extract without polymer (TMEWP) at the same dose. In rabbits that were given a normal diet while under anesthesia, both extracts appeared to diminish the size of the infarct, and in hypercholesterolemic rabbits, they both had antiatheromatic and hypolipidemic effects. For TMEWP and NMF, the reduction in total cholesterol levels was 47% and 88%, respectively[24].

The beneficial benefits of CMG on peripheral and aortic blood pressure hemodynamics in hypertensive patients were established in a randomized double-blind case-controlled crossover design, hinting potential downregulation of the proteasome system and the NADPH oxidase 2 pro-oxidant pathway. The subjects consumed 2800 mg of CMG orally (four tablets of 700 mg or a placebo), and they had evaluations during two subsequent visits spaced by 1 wk[25]. Another pilot investigation also suggested that CMG powder may play a role in human *in vivo* hepato- or cardioprotection. In the group consuming daily 5 g of mastic powder for 18 mo, a reduction in blood total cholesterol, LDL-C, total cholesterol/HDL ratio, lipoprotein (a), apolipoprotein A-1, apolipoprotein B, serum glutamyl oxaloacetic transaminase, serum glutamic pyruvic transaminase, and -glutamyl transferase levels was seen[19]. Since apolipoprotein A-1 is a major component of the HDL-C complex (protective fat removal particles), and thus acts as a cardioprotective molecule, the above observed reduction in the study by Triantafyllou *et al*[19] has to be translated carefully to daily clinical practice especially in patients with increased cardiovascular risk.

EFFECTS OF CMG ON GLUCOSE METABOLISM

The antidiabetic benefit of CMG is a recent discovery, and there is not a lot of evidence to back it up yet. Triantafyllou *et al*[19] presented the first concrete proof of glucose-lowering activity, showing that in the low-dose group, male patients' glucose levels were markedly reduced. According to Georgiadis *et al* [17], CGM had an unexpectedly strong antidiabetic effect, significantly decreasing blood sugar levels in both the low- and high-dose groups of mice. It is noteworthy to note that, in line with Triantafyllou *et al* [19], they found that the low-dose group performed better than the high-dose group. According to a recent study, CMG consumption had positive benefits on blood lipid indicators and insulin resistance in healthy Japanese men. More particularly, 30 min of additional activity three times per week enhanced the effect of the mastic powder intake on insulin, which was lowered by 5 g/d for 6 mo[26].

CONCLUSION

CMG has a wide spectrum of antimicrobial, antioxidant, hypolipidemic, anti-inflammatory, and antidiabetic activities. Several studies have shown that CMG exerts beneficial effects on lipid and glucose metabolism. However, further studies are required to clarify the formula and the active compounds of CMG that have potential cardioprotective effects as well as their use in clinical practice.

FOOTNOTES

Author contributions: Papazafiropoulou AK contributed to writing the manuscript.

Conflict-of-interest statement: The author reports no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: Kerr C
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 926-939

DOI: 10.4239/wjd.v13.i11.926

ISSN 1948-9358 (online)

MINIREVIEWS

Advances in neovascularization after diabetic ischemia

Yue Cai, Guang-Yao Zang, Yan Huang, Zhen Sun, Li-Li Zhang, Yong-Jiang Qian, Wei Yuan, Zhong-Qun Wang

Specialty type: Cardiac and cardiovascular systems

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Amin A, United Arab Emirates; Hettiarachchi P, Sri Lanka; Jovandaric MZ, Serbia

Received: August 5, 2022
Peer-review started: August 5, 2022
First decision: August 20, 2022
Revised: September 9, 2022
Accepted: November 2, 2022
Article in press: November 2, 2022
Published online: November 15, 2022

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Abstract

With the high incidence of diabetes around the world, ischemic complications cause a serious influence on people's production and living. Neovascularization plays a significant role in its development. Therefore, neovascularization after diabetic ischemia has aroused attention and has become a hot spot in recent years. Neovascularization is divided into angiogenesis represented by atherosclerosis and arteriogenesis characterized by coronary collateral circulation. When mononuclear macrophages successively migrate to the ischemia anoxic zone after ischemia or hypoxia, they induce the secretion of cytokines, such as vascular endothelial growth factor and hypoxia-inducible factor, activate signaling pathways such as classic Wnt and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) pathways, trigger oxidative stress response, activate endothelial progenitor cells or enter the glycolysis or lactic acid process and promote the formation of new blood vessels, remodeling them into mature blood vessels and restoring blood supply. However, the hypoglycemic condition has different impacts on neovascularization. Consequently, this review aimed to introduce the mechanisms of neovascularization after diabetic ischemia, increase our understanding of diabetic ischemic complications and their therapies and provide more treatment options for clinical practice and effectively relieve patients' pain. It is believed that in the near future, neovascularization will bring more benefits and hope to patients with diabetes.

Key Words: Diabetes mellitus; Angiogenesis; Arteriogenesis; Ischemia; Hypoxia

Core Tip: This review aimed to give an overview of neovascularization in patients with diabetes. First, we introduced the basic concepts and influencing factors of neovascularization, including angiogenesis and arteriogenesis. Second, the mechanisms regarding cytokines, classical and novel signaling pathways, glycolysis and lactic acid process and so on described in detail. Then, the neovascularization after diabetic ischemia was further described in combination with the complications of diabetes, such as diabetic atherosclerosis, diabetic retinopathy, diabetic nephropathy and diabetic foot ulcer. Last but not least, the treatment plans listed, with advantages and disadvantages, that may offer more treatment options.

Citation: Cai Y, Zang GY, Huang Y, Sun Z, Zhang LL, Qian YJ, Yuan W, Wang ZQ. Advances in neovascularization after diabetic ischemia. *World J Diabetes* 2022; 13(11): 926-939
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/926.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.926

INTRODUCTION

Diabetes mellitus is a complex, heterogeneous, whole-body chronic metabolic disease; it is predicted that by 2045, the number of patients with diabetes, aged 20-79 years, will increase to 783 million[1]. Diabetes-related complications include microvascular plaque formation, ischemia and hypoxia caused by atherosclerosis. They are characterized by severe arterial ischemia, increased risk of amputation of peripheral artery disease and so on. Promoting neovascularization to restore the blood flow of the ischemic area is conducive to disease outcomes while avoiding risk. Hence, it is an urgent issue for scientific researchers.

NEOVASCULARIZATION AFTER ISCHEMIA

Neovascularization is caused by angiogenesis and arteriogenesis. Angiogenesis refers to the budding of new blood vessels in the vascular bed in an original way to form new vasculature, mainly in the capillaries[2]. Arteriogenesis is the formation of new arteries by expanding lumen diameter and remodeling tube walls to restore blood flow in the ischemic area. The new route gradually disappears when the previously blocked artery is recanalized[3].

Angiogenesis

Physiological angiogenesis mainly occurs in embryo development, endometrial thickening and wound healing; the process depends on the ratio of proangiogenic factors to antiangiogenic factors. Pathological angiogenesis is often triggered in disease states, such as atherosclerosis, tumors, systemic lupus erythematosus, *etc*[4], to form abnormal blood vessels with thinner walls and higher permeability. Angiogenesis includes the following steps: (1) Endothelial cells sprout under the action of angiogenic factors[5]; (2) Pericytes aggregate if their absence leads to increased lumen permeability[6] and vascular instability; and (3) The basement membrane is reconstructed to develop mature and stable blood vessels.

Atherosclerosis can lead to severe complications such as myocardial infarction (MI), resulting in heart failure. Endothelial cells, which sprout under the action of angiogenic factors, significantly improve the patient's recovery, which is the focus of current research.

After MI, blood congestion, thromboembolism and compression of the surrounding tissue, proinflammatory factors are released, leading to impaired endothelial integrity, loss of myocardial cells, endothelial cell damage, increased capillary permeability and secretion of proinflammatory cytokines to activate white blood cells and endothelial cells. This results in the release of a large number of inflammatory factors into the infarcted myocardium, thus promoting myocardial inflammation[7]. The formation of neovascularization in the ischemic infarct area to provide nutrients and oxygen is the key to post-MI repair. Neovascularization in the surrounding area of infarction increases vascular density and extends to the core area of infarction[8], and hypoxia plays an essential role in this process.

The most studied factor is hypoxia-inducible factor-1α (HIF-1α), whose reduced protein expression under high glucose conditions leads to increased MI[9]. In anoxic environments, the HIF-1α/vascular endothelial growth factor (VEGF) pathway acts by releasing angiogenic factors. After MI, reactive oxygen species (ROS) in cardiac fibroblasts increase by about 50%, resulting in mutations in the HIF phenotype marked by scar contraction and dysfunction[10]. HIF activation induces VEGF release, which activates endothelial cells (ECs) through the paracrine mechanism, and can be expressed in ECs to participate in angiogenesis. In addition, angiopoietin-like protein 4 stabilizes VEGF receptor 2/Ca^{2+}-dependent cell adhesion molecule 5 complex to maintain endothelial structural integrity and promote

macrophage transformation into a repair phenotype to enhance boundary region angiogenesis.

Arteriogenesis

Arteriogenesis refers to the growth of new arteries or the derived collateral vessels, mainly involved in the active proliferation of ECs and smooth muscle cells, resulting in lumen enlargement and wall remodeling. It comprises primarily two stages[3]. In the early stage, the diameter of lateral branches and tube walls increases under the influence of fluid shear stress. Subsequently, monocytes promote lumen remodeling by secreting metalloproteinases and cytokines. In addition, M1-type macrophages promote the progression of myocardial inflammation[11]. The release of inflammatory factors under ischemia and hypoxia promotes the progression of inflammation and the occurrence of glycolysis. Under this action, fibroblasts are transformed into ECs, triggering epigenetic modification. ECs and fibroblasts contribute to the formation of arteries[12-14].

Human coronary circulation has an extensive anastomotic network. Even one-third of ordinary people have collateral circulation to cope with MI caused by transient vascular occlusion[15]. Further, 20%-25% of patients with coronary artery disease can prevent MI, improve survival and reduce mortality by promoting normal blood flow through collateral circulation during coronary artery occlusion. Approximately 1 in 5 patients cannot tolerate percutaneous coronary intervention or coronary artery bypass grafting. Therefore, collateral growth promotion is a promising therapeutic strategy targeting arteriogenesis. In the absence of coronary artery disease, these arteries are only 100-200 μm in diameter, and the lumen is impassable. When coronary artery disease causes a major artery occlusion, the collateral arteries are remodeled and the lumen is expanded to 100-800 μm in diameter to serve as part of the major artery[16], which is in line with normal routes and has one to two layers of smooth muscle cells. At the same time, the expansion of the diameter of the tube is accompanied by a decrease in the number of collateral arteries. The myocardial protection of large blood flow is more significant in the collateral arteries than in the new capillaries surrounding the infarct area[17].

Although collateral maturation is essential for preserving cardiac function, the related markers are still lacking[18]. Although the influence of coronary artery collateral formation and prognosis has been controversial at present[19], some studies showed[20] that patients with MI having coronary collateral circulation have more severe stenosis and worse cardiac function. Therefore, from a macro point of view, it is believed that collateral circulation benefits at least one-fifth of patients who cannot undergo percutaneous coronary intervention and coronary artery bypass grafting. Physiological or pathological vascular reconstruction and blood flow redistribution can prevent excessive MI and reduce injury to the body. This therapeutic strategy has broad research prospects and is worth further exploration to benefit patients.

Impact factors

As mentioned earlier, neovascularization is affected by various proangiogenic/antiangiogenic factors. The mechanisms and roles of proangiogenic factors, such as HIF, macrophages, VEGF family, noncoding RNA and hepatocyte growth factor, and antiangiogenic factors, such as thrombospondin-1 and interleukin 12 (IL-12), summarized in this study (Tables 1 and 2)[7,21-44].

MECHANISMS OF NEOVASCULARIZATION AFTER DIABETIC ISCHEMIA

Glycolysis

Studies have shown that 80% of ATP in ECs is produced by glycolysis[45], mainly due to the limited number of mitochondria. The energy generated needs to be supplied to the distal tissues. In addition, the ATP generation by glycolysis is faster than that by oxidative phosphorylation. Therefore, glycolysis is the primary energy supply for ECs in normal and hypoxic states. Hypoxia or lack of nutrition can promote the production of VEGF, fibroblast growth factor and other angiogenic factors[46], the concentration and the activity of hexokinase and membrane expression of glucose transporter protein 1. The ECs differentiate into specific cells under high VEGF levels, forming platelet pseudopodia and filiform pseudopodia, promoting migration and stem cell proliferation. Stable glycolysis produces lactic acid [47], promoting VEGF expression and inducing angiogenesis under multiple effects.

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) transcription through HIF-1α is induced by hypoxia, which is an essential regulator of glycolysis and can be phosphorylated by activating kinases such as mitogen-activated protein kinase[48]. PFKFB3 can promote the synthesis of fructose-2 6-diphosphate, activate phosphofructokinase 1 and promote glycolysis. The Notch-Delta-like ligand 4 signaling pathway can promote stem cell extension, tip cell growth and blood vessel germination[49].

Some intermediate products of glycolysis enter the pentose phosphate pathway. The NADPH produced during this process is necessary for nitric oxide (NO) biosynthesis, promoting angiogenesis. When macrophage metabolism shifts to glycolysis, M1 proinflammatory macrophages facilitate glycolysis, interrupting the tricarboxylic acid cycle and producing lactic acid rather than metabolizing pyruvate to acetyl-CoA. Anti-inflammatory M2 macrophages inhibit the pentose phosphate pathway.

Table 1 Mechanisms and functions of proangiogenic factors

Trigger factors	Mechanism	Function	Ref.
HIF	When hypoxia occurs, HIF-α dimerizes with HIF-β, binding to hypoxia response elements in the nucleus, transcribing thousands of genes and promoting angiogenesis	Promote angiogenesis and increase vascular density; stimulate collateral vessel compensatory formation; regulate EPO and other downstream factors; mobilize endothelial progenitor cells	[21-24]
Macrophages	Macrophages are divided into M1 type, which is proinflammatory and phagocytic, and M2 type, which is anti-inflammatory and promotes angiogenesis	Transform into perivascular cells to control vascular permeability; remodel extracellular matrix to provide conduit for apical cells and promote blood vessel germination; endothelial cells and trim abnormal blood vessels	[25-30]
Monocytes	Angiogenesis is directly dependent on the number of circulating monocytes	Induce HIF-mediated release of chemokines and growth factors to stimulate angiogenesis; express angiogenin receptor Tie-2 and exacerbate inflammation	[7, 31]
VEGF family	VEGF-A regulates angiogenesis, vascular permeability and inflammation. VEGF-B regulates angiogenesis and apoptosis. VEGF-C and VEGF-D regulate lymphangiogenesis, apoptosis and fiber formation	VEGF activates a variety of downstream signaling pathways and promotes the proliferation, migration and vascular remodeling of ECs; activate ERK1/2 and promote angiogenesis	[32-34]
Noncoding RNAs	Many noncoding RNAs regulate complex processes of angiogenesis	MiR-25-3p enhances endothelial permeability and angiogenesis. MiR-590-5p subtype NF90 has angiogenic effects	[35, 36]
HGF	Stimulates angiogenesis by inducing endothelial cell proliferation, migration and tubular blood vessel formation	HGF significantly increases the expression of VEGF, decreases the activation of NF-κB and vascular leakage, promotes angiogenesis, is anti-inflammatory, is anti-oxidative and reduces vascular permeability	[37]
Angiotensin II	Angiogenesis is induced by activation of angiotensin 1 receptor and nicotinamide adenine dinucleotide phosphate oxidase	Induction of angiotensin II synthesis can lead to a proangiogenic state	[38]
asTF	asTF is widely expressed in macrophages and neovascularization in AS plaques	asTF affects all key stages of angiogenesis, including proliferation, migration and differentiation and induces increased levels of HIF and VEGF to promote angiogenesis	[39]
Classical Wnt pathway	Under the influence of Wnt factor, β-catenin isolates and enters the nucleus, binding to TCF/LEF and initiating transcription of downstream genes	The Wnt pathway promotes angiogenesis by regulating endothelial cell proliferation	[40, 41]

AS: Atherosclerosis; asTF: Alternatively spliced tissue factor; EC: Endothelial cell; EPO: Erythropoietin; HGF: Hepatocyte growth factor; HIF: Hypoxia inducible factor; MiR: MicroRNA; NF-κB: Nuclear factor kappa-B; VEGF: Vascular endothelial growth factor.

Table 2 Mechanisms and functions of the antiangiogenic factors

Inhibitory factor	Mechanism	Function	Ref.
Platelet reactive protein-1 (TSP-1)	TSP-1 levels increase under hypoxia	TSP-1 inhibits angiogenesis by stimulating endothelial cell apoptosis and inhibiting endothelial cell migration and proliferation as well as inhibiting VEGF and eNOS	[42, 43]
IL-12	IL-12 stimulates the expression of proinflammatory and antiangiogenic genes in monocytes	Neutralization of IL-12 can enhance angiogenesis in ischemic areas and reduce body dysfunction	[7, 44]
Noncoding RNAs	Noncoding RNA has the dual role of promoting and inhibiting angiogenesis	Upregulation of MiR-15a and MiR-16 reduce Tie2 protein levels and inhibit angiogenesis	[35, 36]

eNOS: Endothelial nitric oxide synthase; IL-12: Interleukin 12; MiR: MicroRNA; TSP-1: Thrombospondin-1; VEGF: Vascular endothelial growth factor.

Lactylation

Lactic acid has long been considered as a metabolic waste. Recent studies have shown that lactic acid can be expressed as a signaling molecule in wound healing and angiogenesis[50]. Lactic acid can modify histones to regulate macrophage polarity and expression of tissue repair genes such as arginase-1[51]. In inflammatory diseases such as atherosclerosis, macrophages secrete proinflammatory cytokines such as tumor necrosis factor γ and interleukin 12 to cause extensive damage to surrounding tissue, and anaerobic glycolysis causes lactic acid accumulation[51]. The macrophage polarity transition is a hallmark of the disease, and lactic acid converts macrophages from a proinflammatory phenotype into a repairing phenotype, removes cell debris and promotes wound healing. Inflammatory macrophages undergo modifications, which promote the repair characteristics of macrophages in response to inflammatory damage[52]. In addition, lactic acid can covalently couple with various histone lysine residues during histone acetylation to promote the transcription of homeostasis-related genes. In the late stage of

lactic acid and histone lactate modification and accumulation, the cells switch to a steady-state phenotype, in which inflammatory genes are difficult to induce.

Studies have shown that lactic acid can promote the secretion of VEGF, activate the nuclear factor kappa-B/C-X-C motif chemokine 8 pathway and stabilize HIF-1α, playing a role in promoting angiogenesis signaling molecules. The overall function of lactic acid is to transform the inflammatory phenotype of macrophages into a repair phenotype. We hypothesized that lactic acid was associated with angiogenesis. However, the integration mechanism of lactic acid and hypoxia, such as HIF, chromatin remodeling and other processes extending to angiogenesis, is still unclear and needs further research (Figure 1).

Oxidative stress

When the body is subjected to various diabetic stimuli, the mitochondria is stimulated to produce superoxide, leading to the formation of the powerful oxidant nitrite, which damages DNA and depletes intracellular NAD (+)[53], resulting in a pathological state. Two common mechanisms[54] contribute to increased oxidative stress[55] in diabetes: One is an increase in free radical production and the other is a decrease in the levels of protective endogenous antioxidants. Also, natural antioxidants include dandelion[56], saffron[57,58], hawthorn[59], vitamin C and vitamin E[60]. However, rhizoma polygonate in traditional Chinese medicine can dephosphorylate DNA to damage DNA[61]. In addition, hyperglycemia activates nuclear factor kappa-B, which can lead to changes in the inflammatory response, upregulation of COX-2, inducible NO synthase (NOS)[62], tumor necrosis factor α and interleukin 1, promotion of cell proliferation and inhibition of cell death. The increased expression of inducible NOS catalyzes the production of large amounts of NO[63]. The inhibition of TLR2/4 signaling can avoid nuclear factor kappa-B translocation, ultimately reducing cell apoptosis[64]. The hyperglycemic environment can stimulate the mitochondrial respiratory chain to produce a large number of oxygen free radicals, activate protein kinases C[65] and promote the NADPH-related processes of oxidative stress, leading to endothelial cell apoptosis. A small number of ROS can maintain normal physiological function[66]; however, an excess of ROS causes oxidative stress[67], which can activate multiple stress kinases and related proteases and affect their activities[68], aggravate cytotoxicity and attack cells, leading to endothelial progenitor cell senescence, apoptosis and inhibition of migration and proliferation. Superoxide anions and H_2O_2 in the ROS family play a major role in this process. In addition, the activity of endodermal NOS is reduced, the metabolism of tetrahydrobiopurine (BH4) is abnormal, and dihydrobiopterin (BH2) cannot be recovered in diabetes, resulting in a lower level of BH4[69]. NOS induces the formation of many superoxide anions instead of NO, aggravating oxidative stress. Advanced glycation end products lead to an imbalance in ROS production and clearance and increased endothelial permeability[70]. Oxidative stress impairs angiogenesis through multiple mechanisms.

Endothelial progenitor cells

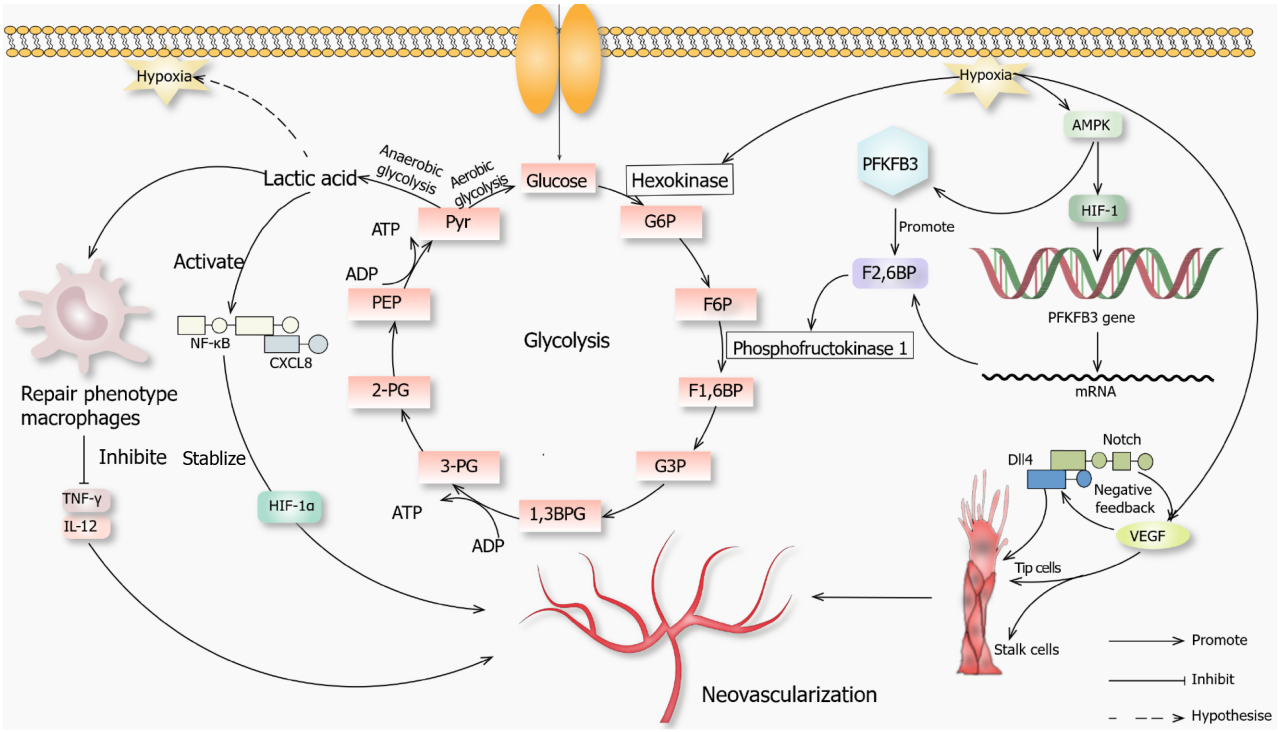
Endothelial progenitor cells (EPCs) are fusiform cells with limited proliferative capacity in the early stage and cells with high proliferative capacity in the late stage[71]. During tissue ischemia, EPCs can be mobilized from bone marrow to damaged blood vessels for vascular repair or angiogenesis, or the number of circulating EPCs increases *via* various factors, including VEGF, stromal cell-derived factor-1 or stem cell factor. EPCs can proliferate, migrate, adhere and differentiate into ECs, repair damaged ECs and secrete angiogenic factors such as VEGF to promote angiogenesis in ischemic tissues[72].

In diabetes, endothelial dysfunction and delayed angiogenesis promote the occurrence and development of diabetic vascular complications. In the high glucose environment, the number of EPCs is reduced and their functions are impaired[73]. In addition, EPCs are less responsive to ischemia, VEGF, stromal cell-derived factor-1 and other stimuli, and the mobilization mechanism is damaged. EPCs may also secrete antiangiogenic factors. The high glucose environment leads to the excessive production of ROS, excessive activation of NADPH oxidase and a significant decrease in the levels of manganese-containing superoxide dismutase and other antioxidant enzymes, resulting in EPC dysfunction[74]. The excessive production of ROS significantly increases the levels of oxLDL, inhibits Akt phosphorylation and endothelial NOS (eNOS) expression, decreases NO activity, inhibits the PI3K/Akt/eNOS signaling pathway and induces the apoptosis of EPCs, migration of EPCs and formation of functional defects in the lumen[75]. In addition, the severe inflammatory environment of diabetes causes impaired adhesion and proliferation of EPCs as well as neovascularization.

However, when patients with diabetes suffer from vascular complications, the number and function of EPCs in different parts (microvessels and large vessels) are different. For example, when such patients suffer from peripheral artery disease, the number of EPCs decreases. However, the proliferative capacity of EPCs is increased in patients with proliferative retinopathy.

Slit2/Roundabout 1/PI3K/Akt/VEGF signaling pathways

Endothelial cell-derived Slit2 plays a proangiogenic role in EC migration and lumen formation through its Roundabout 1 (Robo1) receptor. High glucose levels directly induce Slit2 production or Slit2/Robo1 binding. Robo1 inhibits the activation of the PI3K/Akt pathways and HIF-1α/VEGF signaling pathways and inhibits angiogenesis. PI3K inhibitors also inhibit the HIF-1α/VEGF signaling



DOI: 10.4239/wjd.v13.i11.926

Figure 1 Novel mechanisms of neovascularization. When glucose enters the cell through channels that initiate glycolysis in an aerobic environment, when the anaerobic glycolysis, lactic acid can cause macrophages to convert to a repair phenotype. These macrophages will not produce inflammatory factors such as tumor necrosis factor γ and interleukin 12, which promotes angiogenesis. Lactic acid can activate the nuclear factor kappa-B-C-X-C motif chemokine 8 pathway, promote vascular endothelial growth factor (VEGF) secretion, stabilize hypoxia-inducible factor-1α (HIF-1α), and promote angiogenesis. When hypoxia occurs in the environment, it promotes hexokinase, which promotes glycolysis, and AMPK, which promotes hypoxia-inducible factor-1. It then acts on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3. AMPK also acts directly on 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, and they jointly promote fructose-2,6-biphosphatase, which acts on phosphofructokinase 1 and promotes glycolysis. Hypoxia also promotes VEGF secretion, and a high concentration of VEGF activates the Notch-DLL4 pathway, directly or indirectly promoting the proliferation of tip cells. VEGF also promotes the growth of stalk cells, and both promote angiogenesis. 1,3BPG: 1,3 diphosphoglycerate; 2-PG: 2-phosphoglycerate; 3-PG: 3-phosphoglycerate; F1,6BP: Fructose 1,6-diphosphate; F2,6BP: Fructose 2,6-diphosphate; F6P: Fructose 6 phosphate; G3P: Glyceraldehyde-3-phosphate; G6P: Glucose 6-phosphate; PEP: Phosphoenolpyruvate; Pyr: Pyruvic acid.

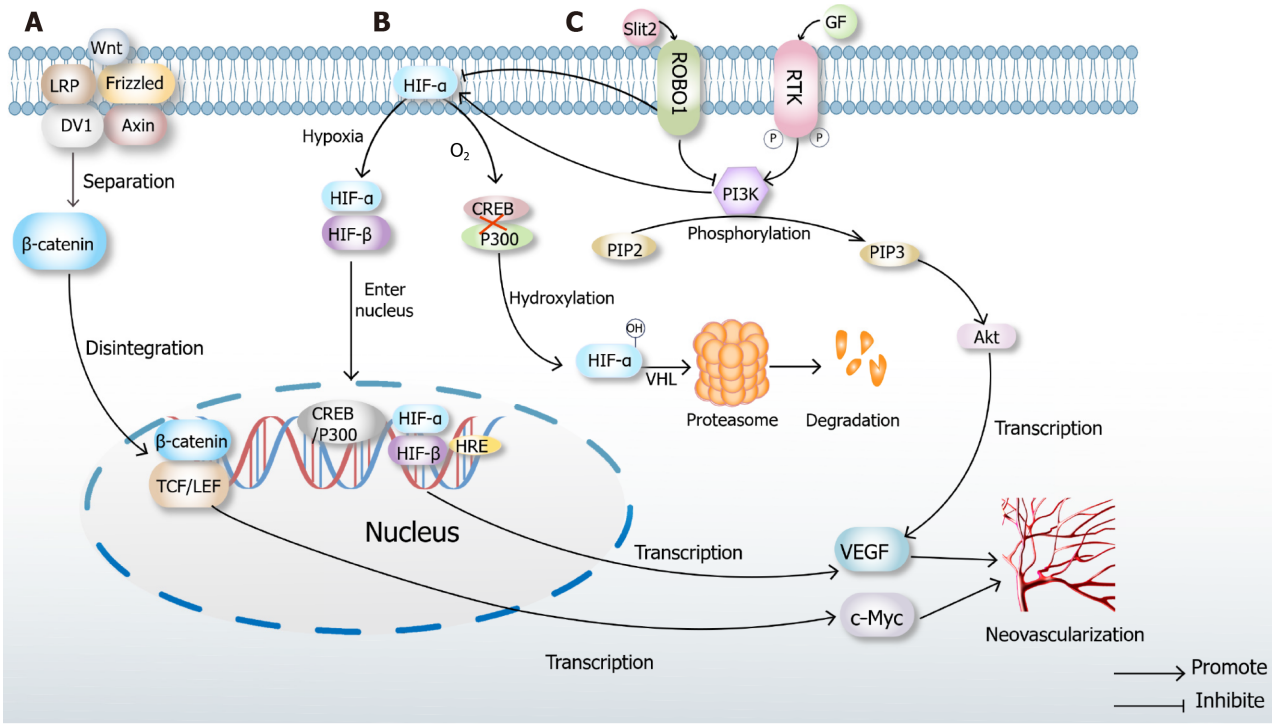
pathway. Hence, Robo1 may be a potential therapeutic target in diabetic ischemic complications with abnormal angiogenesis, such as diabetic nephropathy and diabetic retinal disease[76]. The interference of this signaling pathway can inhibit angiogenesis (Figure 2).

NEOVASCULARIZATION AFTER DIABETIC ISCHEMIA

Diabetes atherosclerosis

Atherosclerotic plaques lead to local hypoxia and trigger angiogenesis of the outer membrane of the vessel. The newly formed vessels are immature and leaky due to the lack of a tight connection between the pericellular cells and ECs. Lipids, inflammatory factors and red blood cells invade the plaques, leading to vessel rupture within the plaques. Proteolytic enzymes released by neovascularization promote inflammatory cell infiltration, and inflammatory factors trigger angiogenesis[77]. At the same time, intraplaque bleeding leads to a rapid increase in plaque volume, the fibrous cap changes to a contraction phenotype, and the fibrous cap becomes thinner[78], increasing the risk of plaque rupture [79]. However, Brezinski *et al*[80,81] put forward an opposite view that plaque rupture is caused by insufficient angiogenesis. The axial extension of neovascularization allows the healing of most plaques, while insufficient angiogenesis cannot sustain the growth of ECs in long necrotic plaques, resulting in plaque rupture and acute coronary syndrome.

Under high glucose conditions, the senescence and death of EPCs are accelerated, the PI3K/Akt/eNOS signaling pathway is inhibited, advanced glycation end products are increased, and fibrin formation is accelerated[82,83]. This triggers endoplasmic reticulum stress and oxidative stress, resulting in insulin resistance and accelerated atheromatous plaque formation. The endothelial cell differentiation is blocked, and angiogenesis is damaged (Figure 3).



DOI: 10.4239/wjd.v13.i11.926

Figure 2 Classical mechanisms of neovascularization. A: Classical Wnt pathway. When there is no Wnt signal, LRP, Frizzled, DV1 and Axin are closely combined with β-catenin. In the presence of Wnt signaling, the complex disintegrates, and β-catenin enters the nucleus, binds to TCF/LEF, transcribes multiple downstream signals such as c-Myc and ultimately promotes neovascularization; B: Under normoxic conditions, hypoxia-inducible factor (HIF)-α inhibits the binding of cAMP-response element binding protein (CREB) and P300, which causes hydroxylation of HIF, then leads to proteasome degradation and inactivation after VHL ubiquitination. When hypoxia occurs, prolyl hydroxylase domains become inactive, allowing HIF-α to migrate to the nucleus, where it dimerizes with HIF-β. The dimer binds to the hypoxia response elements of specific genes in DNA, transcribes thousands of genes such as vascular endothelial growth factor (VEGF) and promotes neovascularization; C: High glucose induces Slit2/Roundabout 1 (Robo1) binding, and Robo1 inhibits the activation of phosphatidylinositol 3kinase (PI3K) /protein kinase B (Akt) and HIF-1α/VEGF signaling pathway and inhibits angiogenesis. PI3K inhibitors also inhibit the HIF-1α/VEGF signaling pathway. After activation of the PI3K/Akt signaling pathway, a variety of cytokines including VEGF will be transcribed to promote angiogenesis.

Diabetic retinopathy

Diabetic retinopathy (DR) can be divided into early nonproliferative DR and late proliferative DR in terms of progression, leading to pathological retinal angiogenesis[84]. Neovascularization extends along the surface of the retina into the vitreous cavity. Still, such vessels are fragile and easily broken, easily leading to vitreous hemorrhage, retinal detachment or macular nonperfusion and related photoreceptor dysfunction[85]. The earliest change is the thickening of the vascular basement membrane. In a high glucose environment, the basement membrane hardens and changes the elasticity of blood vessels, affecting the retinal blood flow and the dynamic balance between the inside and outside of blood vessels[86].

After the blood glucose level increases, the blood-retinal barrier degrades briefly in a few days or weeks, and then Muller cells are activated. Pericytes are lost in about 2 mo, followed by ECs, leading to vascular degeneration 6 mo after diabetes[85]. Vascular degeneration is caused by the loss of pericytes that control vascular patency, resulting in decreased vascular patency, vascular blockage, endothelial cell fusion/degeneration and finally basal membrane dissolution, vascular degeneration and formation of capillaries without ECs. However, under physiological conditions, ECs of retinal capillaries express a high level of tight connections, limiting the circulation of nutrients, soluble factors and cells into tissues [87], and vascular degeneration destroys this structural function.

Consistent with numerous pathological processes, in a hypoxic and ischemic environment, angiogenesis-related factors are promoted, retinal neovascularization occurs, and newly formed blood capillaries migrate to other capillaries and merge to form new blood capillaries[87]. However, the high glucose environment can promote the development of diabetic retinal neovascularization. Proliferative retinopathy is accompanied by tractional retinal detachment and vision loss. Therefore, the treatment aims to inhibit neovascularization. The standard treatment methods include laser therapy and anti-VEGF therapy. The intravitreal injection of VEGF inhibitors has excellent short-term safety. However, whether long-term anti-VEGF may have long-term adverse effects on the function of retinal neurons is unclear.

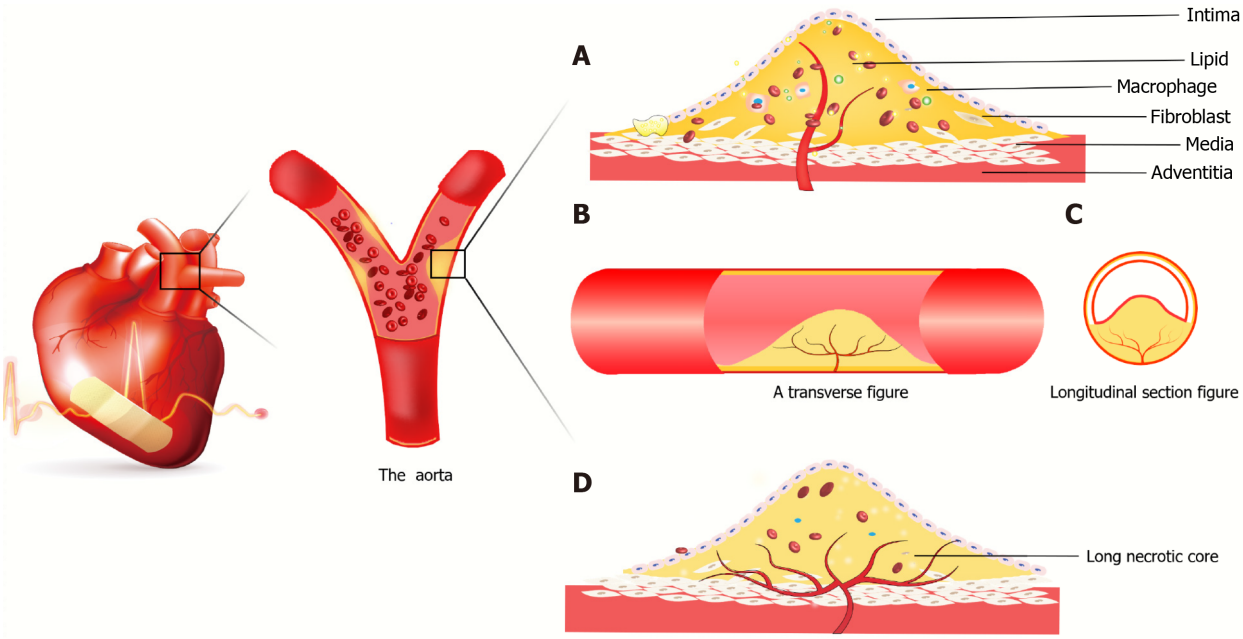


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Figure 3 Atherosclerotic plaque forms in coronary vessels. A: New blood vessels from the outer membrane to the media and intima, macrophages and smooth muscle cells gobble up from the film in lipid foam cell formation. The red blood cells, cytokines, *etc* extend the blood vessels into the plaque. It is because the spin-off of new blood vessels leads to unstable plaques, plaque hemorrhage and rupture resulting in acute coronary syndrome; B: Axial slice of intravascular plaque and neovascularization grows axially in the plaque; C: Transverse view of intravascular plaque; D: It is believed that neovascularization grows in the axial direction in plaques. Due to insufficient growth of neovascularization, long necrotic plaques are ruptured due to ischemia and hypoxia in the plaque, resulting in acute coronary syndrome.

Diabetic nephropathy

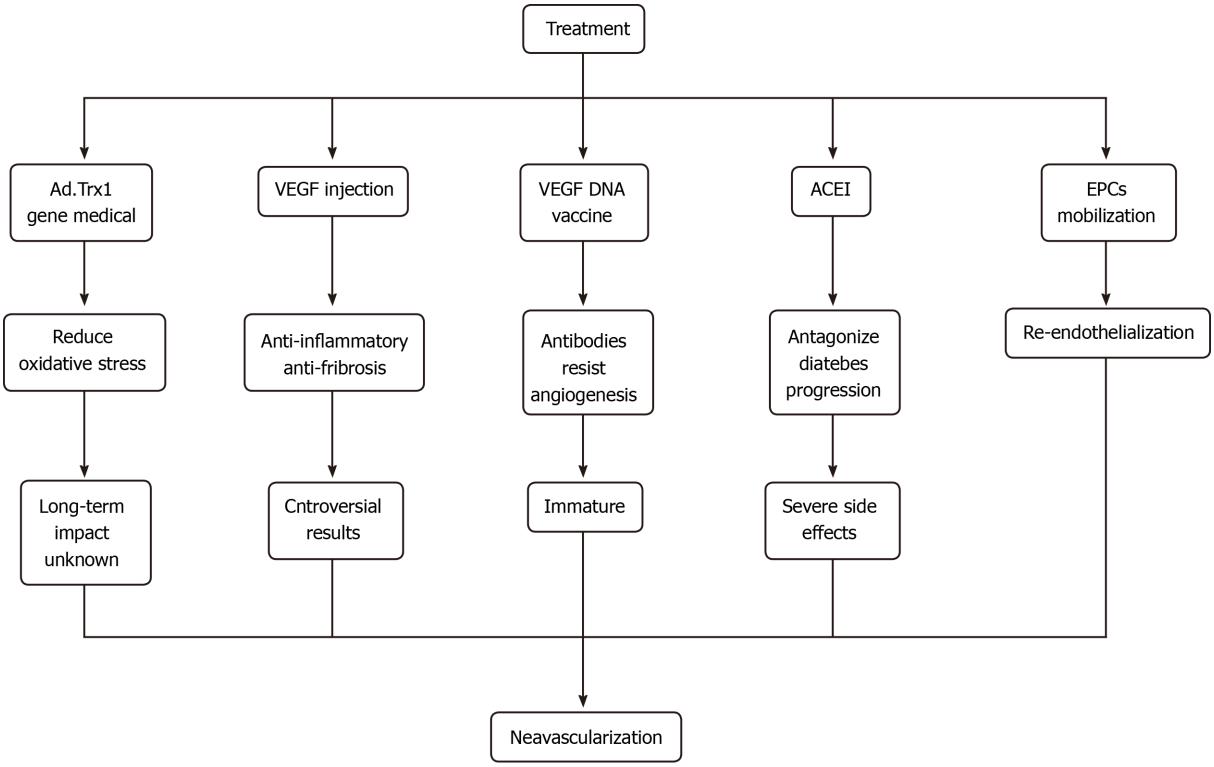
In the early stage of diabetic nephropathy, hypoxia induces HIF generation, and endothelial growth factor or angiopoietin maintains renal vascular density, resulting in increased abnormal angiogenesis, vascular immaturity, plasma protein leakage, increased proteinuria and significantly increased glomerular filtration rate. In the late stage, glomerular capillaries are sparse, and the production of nephrogenic erythropoietin is increased, which aggravates renal hypoxia, and glomerular cells lose vitality[88]. In this process, eNOS activity declines, the utilization rate of NO decreases, oxidative stress abates HIF activation, and VEGF expression is significantly lowered. Also, the levels of antiangiogenetic factors such as platelet response protein 1 and endothelial inhibition significantly increase, the levels of inflammatory cytokines increase, and VEGF expression is inhibited. Further, EPC function is impaired, the inflammatory response is enhanced, and the expression of adherence factors is upregulated, which is accompanied by impaired capillary ECs, endothelial barrier dysfunction and reduced angiogenesis, resulting in abnormal angiogenesis and vascular leakage. Podocytes are an important source of growth factors that regulate endothelial cell proliferation and angiogenesis. Their number increases in the early stage of diabetes and decreases in the late stage. The number of mesangial cells increases, the capillary basement membrane thickens, and the capillary number and area increase, directly or indirectly resulting in glomerular hyperplasia and mesangial expansion.

At present, no precise treatment is available for diabetic nephropathy to inhibit angiogenesis. VEGFA inhibitors can be used, but their levels need to be maintained at an appropriate level *in vivo*[89]. A deviation from moderate levels can cause damage.

Diabetic foot ulcer

Diabetic foot ulcer (DFU) is characterized by neuropathy caused by hyperglycemic levels, arterial stenosis caused by lipid deposition, and ischemic lesions of lower extremities. The DFU healing process can be divided into three overlapping phases: early steady-state and inflammation, arteriogenesis and matrix deposition; mid-late reshaping; and epithelial cell remodeling[90]. Neutrophil granulocyte and macrophages produce cytokines and promote cell proliferation; fibroblasts are rich in collagen fibers and induce angiogenesis and vascularization[91]. Neurological and ischemic lesions lead to impaired healing.

DFU arteriogenesis is reduced in diabetes. Hypoxia and ROS decrease transcriptional activities of HIF, VEGF, angiopoietin 2 and fibroblast growth factor and inhibit collateral development and arteriogenesis after limb ischemia[92]. Hyperglycemic levels result in impaired growth factor production and macrophage function, collagen accumulation inhibition and poor migration and proliferation of



DOI: 10.4239/wjd.v13.i11.926

Figure 4 Cutting-edge approaches to therapeutic angiogenesis. ACEI: Angiotensin converting enzyme inhibitors; EPCs: Endothelial progenitor cells; VEGF: Vascular endothelial growth factor.

keratinocytes and fibroblasts, leading to impaired angiogenesis[91]. At the same time, wound infection reduces the active matrix metalloproteinase 8 (MMP-8) level, increases the active MMP-9 level, inhibits laminin, enhances keratinocyte migration, reduces arteriogenesis and slows wound healing[93].

For the treatment of DFU, the current strategy is to increase arteriogenesis, eliminate oxidative stress and ulcer infection[94]. The best strategy for DFU treatment is to inhibit MMP-9 without affecting MMP-8, which can reduce inflammation and increase arteriogenesis. Applying prolyl hydroxylase domain inhibitors with clinical potential can stabilize HIF and increase its activity to promote arteriogenesis. Mesenchymal stem cells produce growth hormones that drive arteriogenesis and re-epithelialization.

TREATMENT

For treating diabetic ischemic neovascularization, the most crucial way is to control blood glucose levels. On this basis, various therapeutic methods, including gene therapy and vaccine research, have been proposed, which have broad prospects. For example, Ad.trx1 gene therapy can stabilize the microenvironment in the myocardium, reduce oxidative stress and cell death and induce neovascularization and maturation. This is a novel treatment that may improve disease progression and patient recovery[95]. Injection of VEGF and hepatocyte growth factor can promote neovascularization and exert anti-inflammatory and anti-fibrotic effects, but gene therapy is still controversial due to its insignificant effect and needs further study.

Antiangiogenesis antibodies can be generated by the intramuscular injection of the VEGF DNA vaccine; however, the technology is still immature, and the efficacy and long-term impact are not apparent. Hence, further clinical research is needed. Also, angiotensin-converting enzyme inhibitors can antagonize inflammation, increase the number of EPCs and improve the mobilization ability in patients with diabetes. However, this treatment causes an irritating cough, bilateral renal artery stenosis and other adverse side effects[96]. In addition, EPC mobilization promotes re-endothelialization, repairs damaged ECs, promotes angiogenesis and restores blood flow[97,98]. It is believed that with further research, more treatments can be developed to benefit mankind (Figure 4).

CONCLUSION

The advantages and disadvantages of neovascularization to the body are based on different environments. For , MI, peripheral arterial disease, coronary collateral circulation and DFU, neovascularization needs to be promoted to restore the perfusion of the ischemic area and reduce body damage. Neovascularization should be inhibited for DR and tumors to reduce the risk of retinal stripping or tumor metastasis. Although the involvement of neovascularization in disease pathogenesis is still not specific, it has huge prospects for treating ischemia in diabetes.

FOOTNOTES

Author contributions: Cai Y, Zang GY, Huang Y and Sun Z designed, collected and interpreted data and drafted, revised and approved the manuscript; Zhang LL, Qian YJ, Yuan W and Wang ZQ interpreted data and drafted, revised and approved the manuscript.

Supported by the National Natural Science Foundation of China, No. 82070455; the Related Foundation of Jiangsu Province, No. BK20201225; the Medical Innovation Team Project of Jiangsu Province, No. CXTDA2017010; and the Postgraduate Research and Practice Innovation Program of Jiangsu Province, No. KYCX20_3051.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Diabetes

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World J Diabetes 2022 November 15; 13(11): 940-948

DOI: 10.4239/wjd.v13.i11.940

ISSN 1948-9358 (online)

MINIREVIEWS

Nutritional supplementation on wound healing in diabetic foot: What is known and what is new?

Andrea Da Porto, Cesare Miranda, Gabriele Brosolo, Giorgio Zanette, Andrea Michelli, Roberto Da Ros

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Basiri R, United States; Rai V, United States; Shalaby MN, Egypt

Received: August 11, 2022
Peer-review started: August 11, 2022
First decision: September 4, 2022
Revised: September 13, 2022
Accepted: October 11, 2022
Article in press: October 11, 2022
Published online: November 15, 2022

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Abstract

Non-healing diabetic foot ulcers (DFU) are the most notable and striking complications of diabetes mellitus. More than 25% of nonhealing DFU can ultimately lead to amputation of the lower extremity within 6-18 mo after the first manifestation of the wound. Although wound healing is complex, nutritional status is crucial in soft tissue repair. Malnutrition is highly prevalent and overlooked in patients with diabetes and chronic wounds. Moreover, to date, we do not have clear recommendations or evidence about the use of nutritional supplements for improving wound healing in patients with DFU. In this article the authors briefly analyzed the current evidence on the use of nutritional supplements of proteins or amino acids, fatty acids, probiotics, vitamins, and trace elements in the wound healing process in patients with DFU.

Key Words: Malnutrition; Supplements; Diabetic foot; Diabetes; Wound healing; Nutritional therapy

Core Tip: Malnutrition is common in patients with diabetes and chronic wounds. To date we do not have clear recommendations or evidence about the use of nutritional supplements for improving wound healing in patients with diabetic foot ulcers (DFU). This paper aimed to evaluate current evidence regarding the use of Nutritional supplementation on wound healing in patients with DFU.

Citation: Da Porto A, Miranda C, Brosolo G, Zanette G, Michelli A, Ros RD. Nutritional supplementation on wound healing in diabetic foot: What is known and what is new? *World J Diabetes* 2022; 13(11): 940-948
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/940.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.940

INTRODUCTION

The Global Burden of Disease study[1], estimated that 131.0 million (1.77%) people worldwide had diabetes-related lower-extremity complications (DRLECs) in 2016, equal to 34% of the diabetes population. Among these patients almost 105.6 million had neuropathy; 18.6 million had foot ulcers, and 6.8 million undergone to amputation. Moreover, it is known than 84% of lower extremity amputations are preceded by a foot ulcer[2], however ulcer prevention is still an overlooked opportunity[3].

In patients with diabetes known risk factors for developing ulcers are peripheral arterial disease, peripheral neuropathy, repeated trauma, history of previous ulcers, and/or amputation. Male subjects, patients with a longer duration of the disease and a low socioeconomic level have the highest risk[4-7].

Non-healing diabetic foot ulcers (DFU) are the one of the most relevant and dangerous complications of diabetes mellitus[2]. A diabetic foot ulcer is defined as nonhealing in the case of a wound area reduction of less than 50% after 4 weeks of standard care[8]. More than a quarter of non-healing DFU may in the end leads to lower extremity amputation within 6-18 mo after the wound's outset[9].

Although wound healing is regulated by complex mechanisms, the outcome of the process is the complete repair of the damaged tissue. It is known that infection, wound depth, size, and duration negatively impact healing process and are therefore associated with poor outcomes and possible amputation[10].

Nutritional status is crucial in wound healing. The presence of a wound has a negative impact on nutritional status due to the metabolic cost of repairing damaged tissue, in addition to nutrient loss through wound inflammatory exudate. Malnutrition is highly prevalent in patients with chronic diabetic foot wounds[11], and specific micronutrient deficiencies are common and associated with impaired wound healing and increase the risk of amputation in people with DFU[12,13]. Malnutrition is known to impair the inflammatory phase of the healing process, reducing fibroblast proliferation and collagen synthesis, increasing the risk of developing infections, decreasing T lymphocyte function, phagocytic activity, complement, and antibody levels[14]**.** Moreover, nutritional defect of polyunsaturated fatty acids (PUFAs) and consequent reduced synthesis of the essential fatty acid-derived resolvins, (crucial regulators of inflammatory phase of wound healing) are particularly deleterious in people with DFU where diabetes itself is associated with an overall decreased production of pro-resolving lipid mediators[15].

Therefore, nutritional interventions can improve DFU healing. Current Australian guidelines recommend the evaluation of nutritional status when progress towards DFU closure is not made[16] and the use of oral nutritional supplements (ONS) when an oral diet is not sufficient to meet nutritional requirements. However, to date, we do not have clear recommendations or evidence on the use of nutritional supplements to improve wound healing in patients with DFU.

This article aimed to evaluate current evidence regarding the use of Nutritional (Proteins, Aminoacids, Omega 3, probiotics, vitamins, and Trace Elements) supplementation on wound healing in patients with DFU (Table 1).

PROTEIN AND AMINO ACIDS

Proteins and amino acids are the most frequent nutritional supplementation used to improve the healing of diabetic foot wounds, starting from the evidence of the positive effects of these compounds in wound healing. We identified four randomised controlled trial (RCT) studies that supplemented with protein or amino acid mixture in patients with diabetic foot. Eneroth 2004[17] supplemented 27 malnourished patients with diabetic foot ulcers with 20 g protein per 200 mL bottle and compared them with a placebo (26 patients). It is unknown whether there is a difference in the proportion of ulcers healed at the end of the study period, or an absolute change in individual parameters of ulcer dimensions, or ulcer area, over time, for those treated, or not treated with an ONS. Secondary outcomes too, amputation and adverse events (death), didn't demonstrate a difference. Armstrong *et al*[18], an RCT with 270 patients, experimented with supplementation with arginine, glutamine, and b-hydroxy-b-methyl butyrate on foot ulcer healing in people with diabetes. No differences were found between groups in wound closure or time to wound healing after 16 wk. Subgroup analysis shows that the addition of arginine, glutamine, and b-hydroxy-b-methyl butyrate in addition to the standard of care can improve the healing of diabetic foot ulcers in patients with the risk of limb hypoperfusion and/or

Table 1 Strengths and Limitations of the main randomised controlled trial that evaluated the effects of specific nutritional supplement on wound healing in patients with diabetic foot ulcers

Ref.	Study design and Number ofparticipants	Nutrient(s) or Supplement Studied	Aim of study	Main results	Limitations
Eneroth *et al*[17], 2004	Double-blind RCT, 53 patients	20 g protein per 200 mL bottle, 1 kcal/mL	To determine the effects of supplementation on wound healing at 6 mo in subjects with DFU	10/27 (37%) participants in the oral nutritional supplement group healed at 6 mo compared with 12/26 (46%) in placebo group	The sample size was small; certainty of the evidence is very low
Armstrong [18], 2014	Double-blind RCT, 270 patients	Arginine,glutamine and β-hydroxy-β-methylbutyrate	To determine the effects of supplementation on proportion of ulcers healed at 16 wk	65/129 (50%) participants in the arginine, glutamine and β-hydroxy-β-methylbutyrate supplement group healed, compared with 65/141 (46%) in the placebo group	Certainty of the evidence is very low; no difference in quality of life, new ulcers, amputation
Wong *et al* [19], 2014	RCT, 27 patients	Amino acid mixture containing (beta)-hydroxy (beta)-methylbutyrate (HMB), arginine and glutamine	Compare pressure ulcer healing rates	Wound area did not decrease significantly in the short term for both groups. The proportion of viable tissues increased within 2 wk on HMB, arginine and glutamine supplementation; pressure Ulcer Scale for Healing scores showed significant improvement within 1 wk of supplementation for the experimental group	The sample size was small. Observation time was short. Amino acid does not appear to reduce wound size but to improve healing process
Basiri *et al* [20], 2022	RCT, 29 patients	Nutritional supplementation (total energy of 500 calories, 28 g of protein, and essential vitamins and minerals) and education	Effects of nutrition supplementation and education on inflammatory biomarkers	The mean plasma concentration of IL-6 significantly decreased in the treatment group	The sample size was small
Basiri *et al* [20], 2022	RCT, 29 patients	Nutritional supplementation (total energy of 500 calories, 28 g of protein, and essential vitamins and minerals) and education	Nutrient-dense formula combined with nutrition education on wound healing	Treatment group experienced a faster wound healing rate (6.43 mm^2 /wk more reduction in the wound area) than the control group. The mean reduction in the wound area during the first four weeks of the study was almost 13-fold greater in the treatment group compared to the control group	The sample size was small
Sipahi *et al* [22], 2013	Retrospective analysis, 11 patients	Beta-hydroxy-beta-methylbutyrate, arginine and glutamine	Wound healing in diabetic dialysis patients	Healing was observed on the wound depth score of 7 (63.6%) patients and on wound appearance score of 8 (72.7%) patients out of 11	Not RCT; the sample size was small
Soleimani *et al*[31], 2017	Double-blind RCT, 60 patients	Omega-3 PUFA	To determine the effects of flaxseed oil omega-3 fattyacids supplementation on wound healing and metabolic profiles insubjects with DFU	Significant decrease in ulcer length, width, and depth	The sample size was small. The authors did not report data on ulcer healing (percentage of ulcers healed), cost of surgery, quality of life, adverse events, development of any new foot ulcers, amputation rate, incidence osteomyelitis from surgery
Mohseni *et al*[37], 2017	Double-blind RCT, 60 patients	Probiotics (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, and Bifidobacterium bifidum)	To determine the effects of oral probiotic therapy on wound healing andmetabolic status in adult patients with diabetes	Significant improvement in length, width, and depth of ulcers in treatment *vs* placebo groups	The sample size was small. The authors did not report data on ulcer healing (percentage of ulcers healed), quality of life, adverse events, development of any new foot ulcers, amputation rate
Gunton *et al* [39], 2021	Double-blind RCT, 19 patients	Vitamin C 500 mg daily in a slow-release capsule	To determine the effects of oral vitamin C supplement on wound healing	Healing at 8 wk was significantly better in the vitamin C group (median 100 v. -14 %, P = 0.041); healing without amputation occurred in all patients in the vitamin C group	The sample size was extremely small
Yarahmadi *et al*[40], 2021	Double-blind RCT, 25 patients	Oral vitamin E and C or placebo	To determine the effects of oral vitamin C and E supplement on wound	Significantly higher wound size reduction in intervention (P = 0.019); significant decrease in	The sample size was small. All patients were treated even with platelet-rich

			healing	prooxidant-antioxidant balance and hs-CRP in the intervention group ($P < 0.05$)	plasma-fibrin glue dressing and vitamin E
Razzaghi *et al*[46], 2018	Double-blind RCT, 60 patients	50000 IU vitamin D supplements every 2 wk	To determine the effects of oral vitamin D supplement on wound healing an on markers of Inflammation and Insulin resistance	After 12 wk vitamin D treatment resulted in treatment Greater Reduction in ulcer length, width, depth, and erythema rate *vs* placebo	The sample size was small
Afzali *et al* [47], 2019	Double-blind RCT, 70 patients	250 mg magnesium oxide	To determine the effects of oral magnesium oxide + vitamin E supplement on wound healing	Beneficial effects on ulcer size after 12 wk of treatment	The sample size was small
Razzaghi *et al*[46], 2018	Double-blind RCT, 57 patients	250 mg magnesium oxide plus 400 IU vitamin E	To determine the effects of oral magnesium oxide supplement on wound healing	Magnesium plus vitamin E supplements for 12 wk reduced ulcer length and depth *vs* placebo	The sample size was small
Momen-Heravi *et al* [48], 2017	Double-blind RCT, 60 patients	220 mg zinc sulfate supplements	To determine the effects of oral zinc supplement on wound healing	Zinc supplementation for 12 wk was associated with significant reductions in ulcer length and width	The sample size was small

RCT: Randomised controlled trial; DFU: Diabetic foot ulcers; PUFA: Polyunsaturated fatty acids.

hypoalbuminemia. Wong *et al*[19], use a mixture of amino acid containing (beta)-hydroxy (beta)-methyl butyrate (HMB), arginine, and glutamine in diabetic patients with pressure ulcers without obtaining wound area reduction , but demonstrates a better proportion of viable tissues on HMB, arginine, and glutamine supplementation group. Recently Basiri *et al*[20], evaluated the effects on wound healing of a treatment regime based on nutritional education in combination with a nutritional supplement (total of 500 kilocalories, 28 g of protein, and essential vitamins and minerals) in a group of patients with DFU getting a faster wound healing rate in treated group. The mean reduction in wound area during the first four weeks was almost 13 times greater in patients treated with nutritional supplement compared to the control group. Moreover, the same authors demonstrated in another publication that nutritional intervention could have favorable effects on controlling inflammation in patients with DFU[21]. Finally, we included in this analysis a retrospective study, from Sipahi *et al*[22] for interesting results in diabetic dialysis patients supplemented with aminoacid (beta-hydroxy-beta-methyl butyrate, arginine, and glutamine) obtaining healing in most of the patients (63.6%). Dialysis always represents a challenge in the healing of the diabetic foot.

In summary, the results of the current studies did not give a clear effect of proteins and amino acids in the healing of diabetic foot wounds, but there is some evidence of efficacy with improvement in the healing process. The nutritional status of the participants was not an inclusion/exclusion criterion in the studies included in the review. As such, the supplement was generally given randomly irrespective of the presence or absence of malnutrition at inclusion), thus, the baseline nutritional status of the participants is independently correlated with the severity of infection, increased risk of poor outcome, poor prognosis after vascular interventions and it even predicts amputation. Furthermore, it is unclear what the minimum duration is required to achieve a measurable impact on the wound once the serum level of a particular nutrient has improved beyond the deficient range.

POLYUNSATURATED OMEGA-3 FATTY ACIDS

Long-chain Omega-3 PUFA and their bioactive metabolites (resolvins, marine, protectin) have long been considered as powerful negative modulators of acute inflammation and/or inducers of its resolution [23], while the role they play in the wound healing process is still largely unknown. Interest is growing in the understanding of the specific effects of PUFA derived metabolites on the cellular and molecular mechanisms involved in the modulation of inflammatory process[24]. The most bioactive PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that can be obtained directly from many dietary sources as fish and algae, or indirectly through the endogenous metabolic conversion of their precursor, as linolenic acid contained in high concentrations in vegetables and nuts.

Data on the effects of omega-3 fatty acids from flaxseed oil on wound healing in human studies are scarce. Additionally, the studies[25-27] that have evaluated the effects of fish oil supplements (rich in omega-3) on wound healing in patients without diabetes have produced mixed results.

Regarding the consequence of omega-3 fatty acids on inflammatory factors, a meta-analysis showed that supplementation with marine-based omega-3 induce a significative reduction of C-reactive protein,

IL-6, and tumor necrosis factor α[28].

McDaniel *et al*[26,27,29] showed that supplementing omega-3 for 4 wk in a healthy subjects resulted in increased production of pro-inflammatory cytokines, including IL-1ß at wound sites representing a potential therapeutic option for people with chronic wounds[26-29].

As recommended in the Expert consensus and guidance of the American limb preservation society on nutrition interventions in adults with diabetic foot ulcers, mono and PUFAs, including EPA, DHA, and arachidonic acid should be considered because they contribute to membrane fluidity, membrane and intracellular signals, and modulation of apoptotic pathways[30] (ALPS Nutrition Interventions in Adults with Diabetic Foot Ulcers Expert Consensus and Guidance).

In a double-blind RCT, Soleimani *et al*[31] evaluated the effects of daily omega-3 fatty acids from flax seeds for 12 wk in 60 subjects with grade 3 DFU according to "Wagner-Meggitt's criteria". Participants were assigned to receive 1 g twice daily of omega-3 PUFA or a placebo for 12 wk. A significant reduction in ulcer width, length, and depth and an increase in insulin sensitivity and total antioxidant capacity was observed in the intervention compared to the control group. As reported by Moore *et al*[32] the study of Soleimani *et al*[31] had several limitations. The sample size was small. The authors did not report percentage of ulcers healed, cost of surgery, quality of life, amputation rate, and incidence of osteomyelitis from surgery.

PROBIOTICS

An emerging treatment line for wound healing is the use of probiotics, defined by the International Scientific Association of Probiotics and Prebiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[33].

The most common probiotics used as supplements include members of the Lactobacillus spp, Bifidobacterium genera[34], strains from other bacterial species (Propionibacterium acidilactici, Lactococcus lactis, Leuconostoc mesenteroides, Bacillus subtilis, Streptococcus thermophilus) and yeasts (Saccharomycesboulardii)[35].

Perioperative supplementation of probiotics demonstrated accelerated skin healing in diabetic rats, probably due to a reduction of the inflammatory response, increased neovascularization, and increased type I collagen deposition. Supplementation with probiotics also prevents weight loss and improves glycemic control in animal models[36].

In a double-blind RCT, the effects of daily probiotics supplementation for 12 wk were examined in 60 patients with DFU with cellulitis on wound healing[37]. Participants were randomly assigned to receive a probiotic capsule or placebo daily for 12 wk.

The study founds improvement in length, width, and depth of the ulcers in patients treated with probiotic supplement compared with placebo. Additionally, compared to placebo, a significant decrease in HbA1c (-0.6% ± 0.5% *vs* -0.2% ± 0.4%), total cholesterol and CRP levels was observed in treated patients. This study had several limitations: the sample size was small; the authors did not report data on ulcer healing, quality of life, adverse events, and amputation rate.

Studies on probiotic use are limited, for this reason more robust evidence are needed to examine the effects of probiotics on wound healing in patients with DFU.

VITAMINS AND TRACE ELEMENTS

Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin that plays a pivotal role in many biological processes involved in wound healing, including collagen formation, regulation of the immune system, and maintenance of cartilage and bones.

The recommended dietary allowance for adults and older is 90 mg daily for men and 75 mg for women**.** Deficiency of Ascorbic Acid has been associated with increased severity of DFU in a prospective study of 131 patients with diabetic foot[12]. Intriguing data derive from case series of people with diabetes and poorly healing lower limb ulcers in whom there was prompt ulcer healing with vitamin C replacement[38].

Recent evidence from some small randomized controlled trials has raised the idea of a possible benefit of vitamin C supplements on wound healing in DFU. An Australian placebo-controlled randomized trial founds that active treatment with Vitamin C 500 mg daily in a slow-release capsule for 8 wk results in a better percentage reduction in ulcer volume in 7 patients with DFU[39].

With similar findings Yarahmadi *et al*[40] demonstrate that supplements of vitamin E (200IU/2 d) and C (250IU/2 d) for eight weeks significantly increase wound healing in patients with non-healing DFU by enhancing the wound healing process and reducing oxidative stress in 13 patients treated within platelet-rich plasma-fibrin glue dressing plus[40].

Therefore, although seminal evidence is promising and of interest for future research, more convincing and robust evidence on the role of ascorbic acid in wound healing is required.

Vitamin D

Vitamin D is a fat-soluble vitamin that has long been known to help the body absorb certain calcium and phosphorus; both are essential for the building of bone. Circulating levels of 25 (OHD) are low in patients with DFU[12] or patients with severe peripheral artery disease[41]. Furthermore, vitamin D insufficiency has been associated with impaired inflammatory responses, oxidative stress, and wound healing. A meta-analysis of observational studies reports that severe vitamin D deficiency is associated with foot ulceration[42] in people with diabetes. However, evidence of the benefits of vitamin D supplementation in DFU is still lacking. To date, we have only a few RCTs (most of them for the same study group) that evaluated the effects of Vitamin D supplementation on wound healing in DFU. A double-blind, placebo-controlled, randomized clinical trial, conducted among 60 patients with grade 3 DFU according to "Wagner-Meggitt" criteria, showed that after 12 wk of intervention, compared to placebo, vitamin D supplementation resulted in a significant reduction in ulcer length, width, depth, and erythema rate[43]. A very similar study with a comparable number of participants confirmed that a supplement of 60000 IU/weekly of cholecalciferol had a beneficial effect on wound healing when compared with a placebo[44].

More convincing and robust evidence is required on the role of vitamin D supplementation in wound healing.

Trace elements

The nutritional relevance of trace elements has grown rapidly due to a better understanding of their biological functions. They are involved with many enzymatic processes such as catalysis, oxidation-reduction, and cellular transport. Therefore, trace elements have a pivotal role in the synthesis and structural stabilization of both proteins and nucleic acid needed for wound healing. In wound healing, the most important trace elements are zinc, copper, chromium, selenium, and magnesium, which are believed to be essential for the regeneration of physiological tissue in humans[45]. However, only a few randomized controlled trials have tested the effect of trace element supplements on wound healing in DFU.

Effects of magnesium supplements on wound healing in people with diabetes were evaluated in 2 small randomized, double-blind, placebo-controlled trial. The first[46] was carried out among 70 subjects with grade 3 DFU according to the "Wagner-Meggitt" criteria and subjects treated with 250 mg of magnesium oxide had beneficial effects on ulcer size after 12 wk of treatment. In a similar study 57 patients with grade 3, DFU were randomized to take either 250 mg magnesium oxide plus 400 IU vitamin E (n = 29) or a placebo per day (n = 28) for 12 wk. Compared to placebo, taking magnesium plus vitamin E supplements reduced ulcer length and depth[47].

Zinc was tough to improve wound healing due to its effects on insulin resistance, inflammation, and oxidative stress. The effect of zinc supplements on wound healing in DFU was evaluated in a randomized, double-blind, placebo-controlled trial conducted among 60 patients with grade 3 diabetic foot ulcers[48]. Participants were randomly assigned to take either 220 mg zinc sulfate supplements containing 50 mg elemental zinc or a placebo daily for 12 wk. Compared with the placebo, zinc supplementation was associated with significant reductions in ulcer length and width.

CONCLUSION

Current evidence supports that poor nutritional status is common and associated with the impaired healing process in patients with DFU. However, the number of good quality RCTs evaluating nutritional supplementation in DFU patients is limited with varying effects[30,49,50]. Patients with diabetic foot ulcers require good multidisciplinary care to optimize wound healing, but often an important and overlooked aspect of this is their nutritional status. However, evidence for the impact of nutritional interventions on foot ulcer healing in people with diabetes remains uncertain, with studies showing no clear benefit or harm. For this reason, the IWGDF Guidelines on the use of interventions to improve healing of diabetic foot ulcers (2019) recommend not using interventions aimed at correcting the nutritional status of people with diabetic foot ulcers, to improve healing, preferring it as the best standard of care[10]. Instead, according to the new Australian guidelines on wound healing interventions to improve wound healing, the nutritional status of the foot ulcer should be reviewed, and adequate daily nutritional needs should be met as part of the best standard of care "to ensure that this recommendation has not been misinterpreted[15]. In conclusion more and well-designed studies are needed to better clarify the potential impact of nutritional supplementation on the healing of foot ulcers in people with diabetes.

PERSPECTIVES

Author's suggestion for future research and design of proper RCT study: (1) Complete evaluation of nutritional status at the beginning, evidence, and agreement on malnutrition, and evidence if the population examined have a specific nutritional defect; (2) guarantee to all patient's real optimal standard therapy; (3) choose uniformity of wound conditions, don't mix heterogeneous kinds of wounds. Consider that some conditions are time-limited, for example, choosing "infected wounds" we don't define a homogeneous group, with this definition we could include neuropathic, ischemic, traumatic, or mixed lesions with very different characteristics; (4) adequate duration of supplementation and study too, since mean wound healing of diabetic foot is approximately 3 mo, it is not possible to evaluate healing in a shorter period; (5) evaluate evidence of supplementation with blood samples that demonstrate direct change in parameters; and (6) choose multiple parameters, other than healing, to evaluate the effects of supplementation: Rate of wound reduction, percentage of granulation tissue.

FOOTNOTES

Author contributions: Da Porto A and Miranda C came up with ideas and constructs; Da Porto A, Miranda C, and Da Ros R wrote the manuscript; Michelli A, Brosolo G, and Zanette G approved the main conceptual ideas and made corrections; all authors provided final edits and approved the manuscript.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 949-961

DOI: 10.4239/wjd.v13.i11.949

ISSN 1948-9358 (online)

MINIREVIEWS

Combination therapy of hydrogel and stem cells for diabetic wound healing

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Specialty type: Engineering, biomedical

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Jeyaraman M, India; Prasetyo EP, Indonesia

Received: August 28, 2022
Peer-review started: August 28, 2022
First decision: September 12, 2022
Revised: September 25, 2022
Accepted: November 2, 2022
Article in press: November 2, 2022
Published online: November 15, 2022

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Abstract

Diabetic wounds (DWs) are a common complication of diabetes mellitus; DWs have a low cure rate and likely recurrence, thus affecting the quality of patients' lives. As traditional therapy cannot effectively improve DW closure, DW has become a severe clinical medical problem worldwide. Unlike routine wound healing, DW is difficult to heal because of its chronically arrested inflammatory phase. Although mesenchymal stem cells and their secreted cytokines can alleviate oxidative stress and stimulate angiogenesis in wounds, thereby promoting wound healing, the biological activity of mesenchymal stem cells is compromised by direct injection, which hinders their therapeutic effect. Hydrogels form a three-dimensional network that mimics the extracellular matrix, which can provide shelter for stem cells in the inflammatory microenvironment with reactive oxygen species in DW, and maintains the survival and viability of stem cells. This review summarizes the mechanisms and applications of stem cells and hydrogels in treating DW; additionally, it focuses on the different applications of therapy combining hydrogel and stem cells for DW treatment.

Key Words: Combination therapy; Mesenchymal stem cells; Hydrogel; Diabetic wound; Cells delivery; Wound healing

Core Tip: Diabetic wounds are a common diabetes mellitus complication with a low cure rate and likely recurrence. Although stem cell therapy is suitable for diabetic wound healing, simple transplantation methods, such as intravenous, subcutaneous, intramuscular, and local injection, are not conducive to cell survival, thus resulting in compromised efficacy. To improve the outcome of stem cell therapy, researchers have designed different types of hydrogels for stem cell delivery to ensure cell viability and paracrine functions. Herein, we discuss the current roles and applications of hydrogel and stem cell combination therapy for diabetic wound treatment.

Citation: Huang JN, Cao H, Liang KY, Cui LP, Li Y. Combination therapy of hydrogel and stem cells for diabetic wound healing. *World J Diabetes* 2022; 13(11): 949-961
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/949.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.949

INTRODUCTION

Diabetes mellitus (DM) is a significant global public health burden because of its high incidence and mortality rates[1]. In 2019, 1.5 million people died of DM[2]. Diabetic wounds (DWs) are one of the most concerning complications of DM and affect up to 25% of diabetic patients[3]. In addition to causing patient suffering, DW has a low cure rate and high amputation rate, and thus it places a long-term burden on society[4].

DW is difficult to heal because its healing process is unlike that of normal wounds. Normal wound healing typically includes three phases: Inflammation, proliferation, and remodeling. Various cells, growth factors, and cytokines play important roles in each phase to ensure a smooth wound healing progress[5]. Owing to the elevated levels of reactive oxygen species (ROS), impaired immune function, and cellular dysfunction in the DW microenvironment, the healing stage stagnates in the inflammatory phase[6]. In addition, the peripheral arterial disease leads to a lack of blood perfusion and hypoxia within wounds, thereby increasing ROS release[5]. ROS also induces the expression of extracellular matrix (ECM) degradation enzymes that degrade ECM, thus precluding the normal matrix-cell interaction required for wound healing and prolonging the inflammation phase of DW healing[5].

DW healing remains a clinical challenge because of several complications in the DW microenvironment, including oxidative stress, chronic inflammation, and angiogenic dysfunction[7]. Current clinical treatments (standard care) involve glycemic control, offloading, debridement, and infection management, which are painful and insufficient for curing DWs[8]. Therefore, new approaches for improving DW healing must be developed. The application of functional hydrogel dressings or scaffolds is a promising advanced therapy[9].

Hydrogels are three-dimensional (3D) networks with a high water content and have been intensively studied because they can be functionalized and have good biocompatibility. Several studies have shown that hydrogels provide a moist environment, contribute to cell migration and tissue regeneration, and promote wound healing[10]. Therefore, hydrogels are considered ideal dressings for DWs[11]. Furthermore, hydrogels provide antioxidant, antibacterial, proangiogenic, and proliferative functions owing to the sustained release of bioactive agents encapsulated in hydrogels. Stem cells are bioactive agents that promote wound healing and are effective in skin regeneration[12].

Stem cells possess self-renewal and differentiation abilities and are essential for post-injury skin repair[13]. Thus, stem cell therapy has become a promising new approach for treating DWs. Local injection of the cell suspension or stent implantation stimulates neovascularization, accelerates wound closure, prevents wound contracture and scar formation, and ultimately improves wound healing[14]. However, the outcome of stem cell therapy is hindered by the poor bioactivity of stem cells and thus the low amounts of secreted cytokines in the hyperglycemic inflammatory microenvironment of DWs. Effective stem cell delivery remains a challenge[15].

To achieve better healing outcomes combining hydrogel and stem cell treatment is one of the most promising therapies for DWs[16]. Although various reviews on stem cell therapy or hydrogel therapy for DWs have been reported, reviews on combined therapy are limited. Herein, we review the mechanisms of DW therapy combining hydrogel and stem cells and focus on preclinical studies of therapy combining hydrogel and stem cells for DWs.

FUNCTIONAL HYDROGELS FOR DW TREATMENT

Wound dressings play an essential role in DWs[2]. Hydrogels have become appealing and promising among various wound dressings owing to their high moisture retention, biocompatibility, and similarities to living tissues[17]. Hydrogels accelerate wound healing by maintaining gas exchange in

the wound, reducing pain by absorbing exudates, preventing infection, and maintaining a moist environment for cell migration. In addition, hydrogels have been used as delivery systems to minimize drug toxicity and improve drug delivery efficiency[2]. Functional hydrogels, such as antioxidant, immune regulation, and vascularization hydrogels, have been designed according to the wound microenvironment of DWs.

DWs are often accompanied by oxidative and antioxidant imbalance *in vivo*. Hydrogels are designed to alleviate excessive oxidative reactions. Self-antioxidant materials, such as 2-hydroxyethyl methacrylate[18] and polyvinyl alcohol, can directly act on wounds; additionally, gel-loaded antioxidant drugs, such as curcumin[19], or bioactive substances can be used to achieve antioxidant effects. These materials act as reducing agents.

Because the inflammatory phase has an active defense response to external stimuli, the inflammatory response aids in cleaning the wound during the healing process[20]. However, in chronic wounds, such as DWs, owing to repeated tissue damage, cytokines continue to recruit immune cells to the wound, thereby resulting in an excessive inflammatory response and blocked healing[21]. Therefore, the inhibition of excessive immune responses is also considered. Hydrogels, such as sodium alginate and zwitterionic hydrogels, can provide a protective microenvironment for wounds and regulate the transformation of macrophages between proinflammatory and anti-inflammatory[20]. Meanwhile, anti-inflammatory drug-loaded hydrogel dressings have a local sustained-release effect[22]. Responsive hydrogels that can change their properties according to environmental clues to achieve sustained release of entrapped drugs are also desirable.

Angiogenesis is essential for tissue regeneration, whereas the formation of healthy blood vessels is hindered by various microenvironment conditions in DWs[23]. Therefore, promoting blood vessel formation is conducive to DW healing. Studies have shown that some hydrogel materials, such as chitosan and hyaluronic acid, regulate the activity and distribution of cytokines or growth factors[24]. These materials simulate the microenvironment of the ECM, thereby promoting tissue formation. Bioactive components, including epidermal growth factor and vascular endothelial growth factor, can also be encapsulated by hydrogels, which can promote the regeneration of blood vessels[25].

In general, the mechanism of hydrogels in DWs is relatively clear and positively affects DW healing.

CURRENT STUDIES OF MESENCHYMAL STEM CELLS FOR DW HEALING

In addition to selecting different hydrogel materials, drugs, and biological factors, using stem cells to treat DWs is desirable. Stem cells can asymmetrically replicate and differentiate into different cell types [26]. With the unlimited replication capacity, they can provide numerous "sister" stem cells[15]. Furthermore, because stem cells secrete pro-regenerative cytokines, stem cell therapy, which treats diseases or injuries by administering stem cells into damaged tissues, has been used as an intervention for DWs[27]. Stem cells used for wound healing and tissue regeneration include embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells (MSCs)[15].

Allogeneic, xenogeneic, and autologous MSCs have been widely used in skin regeneration and wound healing owing to their significant proliferation, migration ability, and long-term self-renewal potential[28]. Considering the impaired function of MSCs derived from patients with diabetes and the risk of tissue rejection, allogeneic MSCs are more widely used[29].

MSCs that are locally injected into wounds are involved in various stages of wound healing. They reduce inflammatory responses through immunomodulation and growth factor production[15], accelerate neovascularization and epithelialization, and stimulate collagen synthesis[30], thereby accelerating wound healing[30]. Additionally, clinical studies have demonstrated the efficacy of MSCs in treating diabetic ulcers[30]. For example, injecting allogeneic MSCs into the dermis-epidermal junction[31] or subcutaneous and intramuscular tissue around wounds[32] facilitated DW healing in patients.

The potential benefits of MSC therapy have been demonstrated in several studies. Although simple transplantation methods, such as intravenous, subcutaneous, intramuscular, and local injection of MSCs, have achieved some preclinical and clinical success[5], MSC performance still has numerous limitations. Premature senescence and apoptosis of MSCs transplanted in DWs are some of the biggest limitations[33,34]. Owing to hyperglycemia caused by DM, DWs generate a chronic inflammatory microenvironment and accumulate advanced glycation end products, which is not conducive to the survival of stem cells[35] and increases the degradation of growth factors secreted by the effector cells, thus compromising efficacy[36]. Hence, the delivery strategy must be optimized to ensure cell viability, paracrine function, and differentiation function, which in turn ensures MSC therapy outcomes.

Abundant evidence has shown that using hydrogels to deliver MSCs improves DW healing. Hydrogels are ideal carrier systems for stem cells because they produce a relatively uniform distribution of transplanted cells and retain high water content, close to that of the native tissue, thus improving the retention and survival of stem cells at transplantation sites. Transplanted stem cells can exert their functions through paracrine signals and differentiate into the various cell types required in healthy tissues (Figure 1).

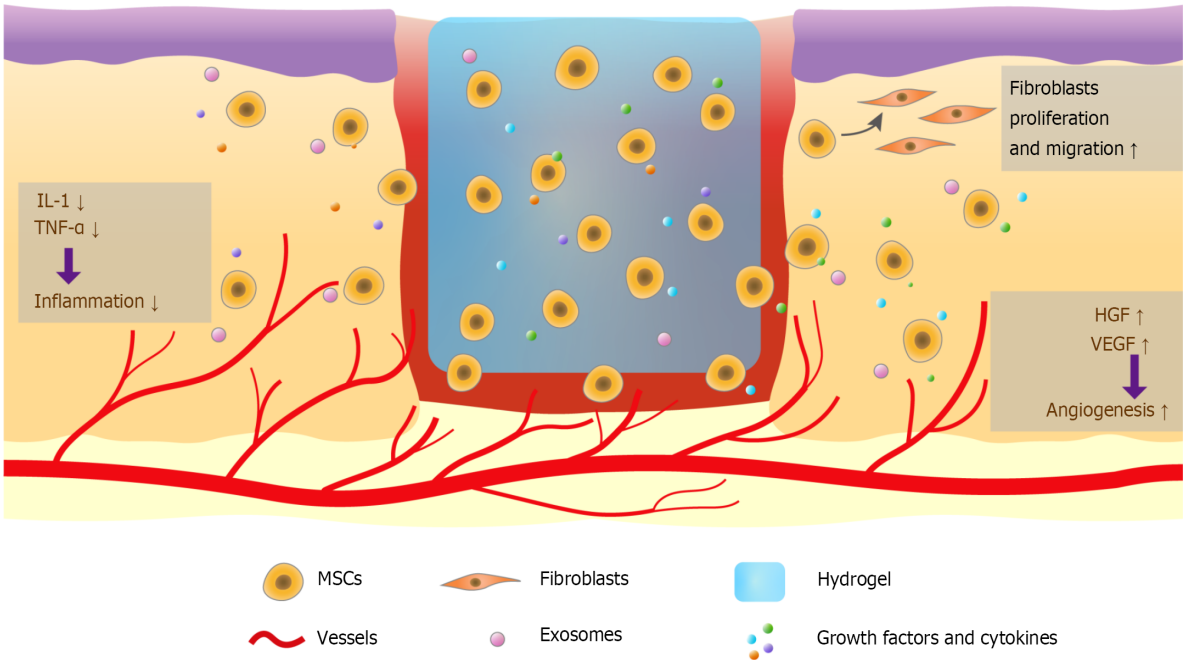

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Figure 1 Therapy combining hydrogels and mesenchymal stem cells promotes diabetic wound healing. Mesenchymal stem cells (MSCs) in hydrogels are long-lasting in the wound and regulate wound healing. These cells release exosomes, growth factors, and cytokines, reduce the levels of interleukin-1, tumor necrosis factor-α, and other pro-inflammatory cytokines to modulate the inflammatory response, enhance angiogenesis *via* increasing vascular endothelial growth factor and hepatocyte growth factor, and promote fibroblast and keratinocyte migration. MSCs can also be transdifferentiated into other cell types to increase wound closure. MSCs: Mesenchymal stem cells; IL-1: Interleukin-1; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor.

APPLICATIONS OF COMBINATION THERAPY OF HYDROGEL AND STEM CELLS FOR DW HEALING

As previously discussed, although stem cell therapy has promising potentials for DW healing, the lack of an optimal delivery strategy is one of the biggest obstacles to its therapeutic efficacy. Traditional injection of MSCs always results in low cell viability and transient engraftment, whereas using advanced biomaterial scaffolds (such as films, nanofibers, and hydrogels)[13] to maintain cellular viability, proliferation, and differentiation has received considerable attention[37]. Hydrogels have physical and biological characteristics similar to those of natural tissues[36]; this renders them as ideal candidates for stem cell delivery. Inspired by the encouraging outcomes of hydrogels on DW healing and their function as a carrier system for drugs, the efficacy of MSCs has been improved with hydrogels [37]. For a successful clinical application of the therapy, the optimal hydrogel composition for cell delivery must be considered, and appropriate application methods to ensure stem cell viability and promote DW healing must be designed. Currently, the most common application methods of hydrogels and stem cell combination therapy for DW healing are divided into hydrogel sheets, *in situ* forming hydrogels, and hydrogel microspheres (MS) (Figure 2).

HYDROGEL SHEETS

Applying hydrogel sheets on wounds is a convenient stem cell delivery method, wherein hydrogels are typically preformed in molds, with stem cells seeded onto or inside hydrogels. Rustad *et al*[38] seeded MSCs onto collagen-pullulan hydrogels and significantly accelerated wound healing and skin appendage recovery in mice within 11 d. The amount of microangiogenesis was approximately doubled in wounds treated with MSC-seeded hydrogel sheets compared with those treated with MSC injection. Given that the biomimetic hydrogel provides a functional niche to augment the regenerative potential of MSCs, the implanted MSCs differentiated into dermal fibroblasts, pericytes, and endothelial cells, which contribute to wound healing[38]. In another study, Guo *et al*[39] demonstrated the improved retention and survival rate of MSCs in hydrogel sheets when transplanted into mouse hearts compared to cell suspension alone. Cells were observed inside the hydrogel sheets for over 9 d in ICR mice.

In vitro culturing of stem cells within hydrogels was found to promote cell adhesion and enhance stem cell functions by supporting normal phenotype maintenance and empowering the transdifferen-

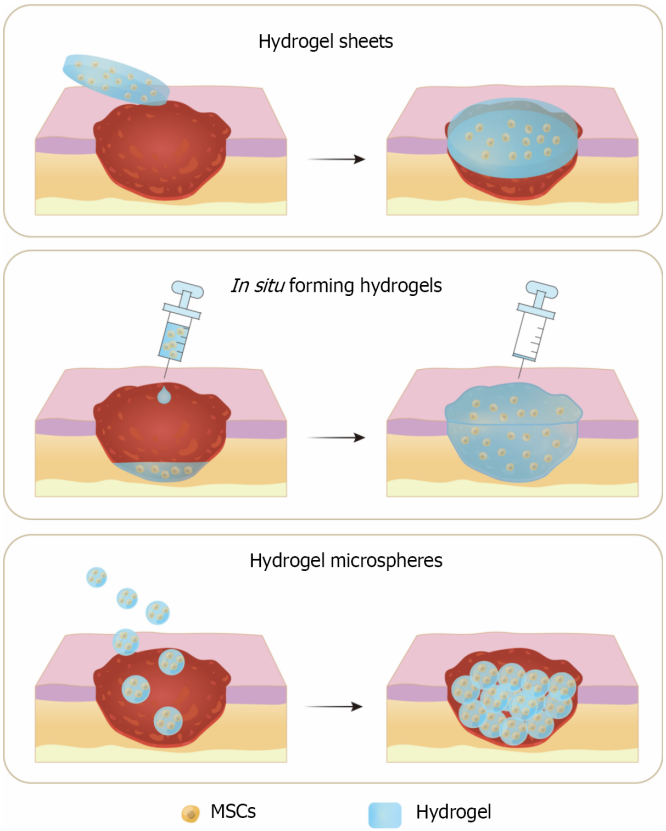

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Figure 2 Three application methods of hydrogels and mesenchymal stem cells combination therapy for diabetic wound healing. A: Hydrogel sheets preformed before application; B: *In situ* forming hydrogels injected at the wound for sol-gel transition; C: Hydrogel microspheres applied onto the diabetic wound. MSCs: Mesenchymal stem cells.

tiation capacity into specific skin lineages compared with the immediate transplantation of stem cell-seeded hydrogels[40]. Da Silva *et al*[41] pre-cultured adipose-derived stem cells (ADSCs) in hyaluronic acid-based sponge hydrogel in neurogenic/standard media for 14 d before transplantation onto the DWs of mice. Wounds treated with pre-cultured ADSCs-loaded spongy hydrogels improved wound closure rates compared to the untreated control and acellular spongy hydrogel groups after healing for 4 wk. The hydrogel sheet promoted the polarization of M1-type macrophages to the M2 type (anti-inflammatory) and improved successful neoinnervation.

Because of the high concentration of inflammatory cytokines in the DW microenvironment, which impairs the activity of MSCs and degrades growth factors secreted by stem cells, single functional hydrogel sheets may not be sufficient for DW healing[42]. To be more suitable for DW treatment, hydrogel sheets that inhibit inflammatory responses or protease activity are more effective[43]. Ahmed *et al*[44] studied the wound healing efficacy of bone marrow-derived mesenchymal stem cells (BMSCs) delivered by nitric oxide (NO)-releasing hydrogels on diabetic rabbits. As an endogenous molecule, NO increased angiogenesis and improved immune responses during acute infections. NO-releasing hydrogels increased the viability and proliferation of BMSCs under oxidative stress. In addition to improving collagen deposition and promoting re-epithelialization and angiogenic activity, the NO-releasing hydrogel with BMSC treatment upregulated the expression of growth and cytoactive factors for DW healing within 16 d[44].

In addition to traditional manufacturing technology, 3D bioprinting builds special structures layer-by-layer according to a predetermined computer model that better fits the skin's architecture and geometry, providing hydrogel sheets with more complex structures[45]. Xia *et al*[46] developed curcumin-incorporated 3D bioprinting gelatin methacryloyl (GelMA) to seed ADSCs and promote DW healing within 21 d. Curcumin encapsulation in 10% GelMA hydrogel exhibited inhibitory effects on ROS generation and ADSC apoptosis, and living cells were detected after scaffolds embedded with ADSCs were implanted into the backs of nude mice for 21 d. Further, the scaffold increased the amount of collagen deposition and induced angiogenesis in DWs[46]. In addition to 3D bioprinting, multifunctional hydrogel sheets with complex 3D structures can be produced by folding or weaving microfiber-shaped hydrogels[47]. Hydrogel sheets can also be easily functionalized, such as the thermally responsive release of stem cells or drugs[48] for oxidative stress resistance, antibacterial activity, and other functions.

Because stem cells can be cultured separately and the hydrogel sheet is easy to handle, combination therapy with hydrogel sheets and MSCs is easily translated into a clinical setting[49]. According to a clinical report, Ravari *et al*[50] applied BMSCs along with platelets, fibrin glue, and bone marrow-impregnated collagen matrix onto wounds, which resulted in the complete wound closure in 3 of 8 patients with aggressive, refractory DWs within 4 wk of treatment. Additionally, topical administration of placenta-derived mesenchymal stem cells in a sodium alginate hydrogel completely healed diabetic foot ulcers[51]. However, this clinical case report must be evaluated further because of the limited sample size of the report. Although functionalizing or changing shapes is very convenient, hydrogel sheets must be pre-formed before application. Because hydrogel sheets are not conducive to long-term storage and the bonding between the sheets and wound surface is limited, *in situ* forming hydrogels have attracted attention.

IN SITU FORMING HYDROGELS

In situ forming hydrogels are another mainstream application of combination therapy, with stem cells suspended in the precursor solution before application[52]. After the mixed precursor solution is injected into the wound site, the hydrogel containing stem cells is formed *in situ* on wound beds *via* chemical bonds[53]. Compared with hydrogel sheets, injectable hydrogels are more flexible in their application; this flexibility allows them to adapt to complex-shaped wounds and fit closely[54]. Eke *et al*[55] designed a precursor solution composed of GelMA and methacrylated hyaluronic acid containing ADSCs, which can be crosslinked within 40 s of ultraviolet irradiation to form hydrogels *in situ*. Reportedly, the hydrogel promoted cell proliferation, and *in vivo* studies revealed a three-fold increase in vascularization for the ADSC-loaded hydrogel group compared to the hydrogels without cells.

However, because ultraviolet irradiation may induce chromosomal and genetic instability[56], ultraviolet-crosslinked hydrogels on exposed wounds negatively affect cell viability and differentiation [57], which is detrimental to wound healing. Owing to its high biocompatibility and specificity[58], enzymatic crosslinking has received considerable attention[59]. Yao *et al*[52] developed a gelatin-hydroxyphenyl hydrogel with the dual enzyme crosslinking of horseradish peroxidase and galactose oxidase, and the hydrogel encapsulated with BMSCs achieved gelation within 5 min at the wound site. The gelatin-hydroxyphenyl hydrogel provides a friendly 3D microenvironment for BMSCs, thereby improving the transplanted cells' survival and accelerating wound closure[52].

Given that frequently studied natural hydrogels, such as gelatin, collagen, or hyaluronic acid, contain a single component of ECM, their potential to provide the optimum microenvironment for stem cell proliferation and differentiation is limited[60]. ECM maintains the original components of the native tissue and is considered an ideal scaffold for tissue regeneration[61]. Chen *et al*[62] developed an ECM-derived hydrogel from human decellularized adipose tissue matrix to deliver ADSCs to DWs. The hydrogel was prepared *via* pepsin digestion and pH neutralization. The paracrine activity of ADSCs encapsulated in the hydrogel was enhanced, whereas the secretion of hepatocyte growth factor increased, thus promoting neovascularization during wound healing[62]. Compared with the untreated control, local ADSC injection, and acellular hydrogel groups, treatment with ADSC-hydrogel composites accelerated wound closure in diabetic mice and restored cutaneous appendages within 14 d [62].

For better DW healing outcomes, specific materials are co-entrapped inside the hydrogel for hemostasis and anti-inflammatory properties, and the stem cell viability in the hydrogel can reach an ideal state by optimizing its mechanical strength. Xu *et al*[63] encapsulated MSCs in an injectable hydrogel system of GelMA and chitosan-catechol cross-linked with dithiothreitol to repair full-thickness DWs. Chitosan-catechol has a good hemostatic effect, and zinc ions were introduced into the hydrogel to enhance angiogenesis. The cell adhesion, proliferation, and differentiation potency of umbilical cord-derived mesenchymal stem cells *in vitro* were well maintained in GelMA with optimal stiffness. At the same time, the hydrogel-umbilical cord-derived mesenchymal stem cells combined treatment promoted DW healing by inhibiting the inflammatory factors TNF-α and IL-1β *in vivo*, with a wound closure rate of 92.2% within 14 d. Compared with the untreated control, local umbilical cord-derived mesenchymal stem cell injection, and acellular hydrogel groups, collagen deposition was significantly abundant on day 7, whereas the most vascular regeneration with the earliest hair follicle formation was found on day 14[63].

Dispersive MSCs are usually loaded inside hydrogels. Recently, 3D MSC spheroids were found to possess better differentiation potential than dispersive MSCs[64], which exhibited enhanced vascularization and anti-inflammatory effects[65], thereby promoting wound closure[66]. Yang *et al*[67] combined injectable thermosensitive chitosan/collagen/β-glycerophosphate hydrogels with 3D MSC spheroids, rapidly converted to a gel by physical cross-linking at body temperature, and then completely covered the wound surface and fitted to any shape of the wound bed. Compared with the local 2D monolayer MSC injection and 2D monolayer MSC-encapsulated hydrogel groups, angiogenic factors were much higher for wounds treated with 3D MSC spheroid-encapsulated hydrogel (almost 3-fold), and neovascularization was enhanced, thereby achieving complete re-epithelialization within 3

wk of implantation[67].

Although *in situ* forming hydrogels adapt to complex-shaped wounds and fit tightly, thus enabling flexible use at the wound bed, the bulk hydrogel formed at the wound site produces poor tissue infiltration and thus low stem cell survival. Compared with *in situ* forming hydrogels, hydrogel MSs have a larger specific surface area and more specific functions, thus playing an essential role in the medical field.

HYDROGEL MS

Hydrogel MSs exhibit good dispersion and stability in physiological environments with a high drug-loading capacity[68]. Their drug-carrying[69] and bioactive factors[70] are highly effective in wound healing. We previously demonstrated that antibiotic and growth factor separately loaded alginate/$CaCO_3$ MSs prepared using microfluidic technology sustainably released drugs and exhibited pH sensitivity. These MSs were embedded in the regenerated tissue and functioned as scaffold materials. They improved wound healing with thicker granulation tissue and stimulated angiogenesis, ideally meeting the requirements of different stages of wound healing[71]. Lei *et al*[70] developed biohybrid agarose MSs conjugated with basic fibroblast growth factor, which achieved local growth factor delivery, stimulated angiogenesis, and enhanced wound healing in diabetic mice.

The special geometry of hydrogel MSs is conducive to the diffusion of nutrients and wastes[72]. MSs that deliver stem cells can release stem cells, thereby promoting proliferation and differentiation of surrounding cells and enhancing the formation of integrated functional tissues[73]. Stem cell-loaded MSs have been applied in various tissue systems, including cartilage[74], bone[75], bone marrow[72], and brain[76]. Intracerebral implantation of stem cells using MSs in the rat brain improved stroke treatment[76]. Mao *et al*[72] demonstrated that microgel encapsulation sustained MSC survival after intravenous injection in mice and enhanced the immunoregulatory capacity of MSCs in a bone marrow transplantation model.

Considering that our previous study demonstrated that hydrogel MSs act as scaffolds and gradually integrate into regenerated skin tissue, we designed gelatin MSs encapsulated with ADSCs from rats (rADSC/MS) with an ideal mechanical strength and degradation rate that matched tissue regeneration to improve DW healing[77]. Gelatin MSs promoted the adhesion and proliferation of fibroblast cells and maintained the viability of encapsulated rADSCs. Slowly released exosomes from rADSCs were eventually internalized by HUVECs, which suggested a potential exosome mechanism for improving wound healing. The implanted rADSC/MS gradually integrated into the regenerated skin tissue, thus facilitating the arrangement of neat collagen fibers. Compared with the untreated group and the MS group, rADSCs embedded in rADSC/MS promoted M2 macrophage polarization and recovery of peripheral nerves, formed larger blood vessels, and eventually generated a dermis close to normal tissue within 14 d[77].

Previous studies have demonstrated that hydrogels provide a functional niche for MSCs, which enhances MSC regeneration potential and promotes wound healing. Preclinical studies on the combined treatment of DWs with hydrogels and stem cells are summarized in Table 1.

CONCLUSION

This review discussed the benefits associated with therapy combining hydrogels and MSCs for DW healing. Researchers have explored different application methods for stem cell delivery with hydrogels, including hydrogel sheets, *in situ* forming hydrogels, and hydrogel MSs. In addition to providing a friendly microenvironment for stem cells, this strategy enhances the adhesion between the dressing and wound and facilitates the function of stem cells, ultimately benefiting vascular and neural regeneration in DWs. Among these application methods, hydrogel MSs have the advantages of a larger specific surface area, more uniform dispersibility, and more specific functions; additionally, they can effectively deliver various types and functions of cells into the wound. Therefore, hydrogel MSs loaded with stem cells are expected to play an important role in clinical practice.

Therapy combining hydrogels and MSCs has shown great potential for DW healing. However, the plasticity of MSCs has led to their double-sidedness for clinical applications. Although the multi-differentiation ability provides them with good application prospects, it increases the risk of tumorigenicity [78]. As a solution, cell-free treatments, such as exosomes and artificial cell products derived from the MSCs secretome have attracted recent interest. Exosomes and secretomes retain the paracrine factors of stem cells[7]. Although extensive studies have explored the combination therapies of hydrogels and MSCs for DW healing, additional work is required to optimize parameters, such as the storage and transport stability of cells, and avoid their tumorigenic and immunogenic risks. Further improvement and testing of this technology *in vivo* will also contribute to the clinical transformation of combination therapy.

Table 1 Summary of studies regarding therapy combining hydrogels and stem cells for diabetic wound healing

Stem cell information, types, dosage in cells/wound	Hydrogel composition	Hydrogel types	Application methods	Animal	Wound size diameter, location	Full re-epithelialization efficiency	Outcome	Ref.
UMSCs from human, xenogeneic, 1 × 10^6	Self-assembled nanopeptide hydrogels based on RADA16-I, RGD, and KLT peptide solutions	Self-assembled nanopeptide hydrogels with easy biomimetic functionalization	Cells were encapsulated into the *in situ* forming hydrogels	NOD/SCID mice	8 mm, dorsal	10 d	Accelerated skin wound healing by inhibiting inflammation and promoting angiogenesis	[14]
BMSCs from rats, allogenic, 2 × 10^5	N-chitosan/ HA-ALD hydrogel	Hemostasis and antimicrobial hydrogels	Cells were encapsulated into the *in situ* forming hydrogels	STZ-induced diabetic rats	5 mm, foot	12 d	Promoted wound healing; stimulated the secretion of growth factors from rBMSCs, and modulated the inflammatory environment by inhibiting the expression of M1 macrophages and promoting the expression of M2 macrophages, resulting in granulation tissue formation, collagen deposition, nucleated cell proliferation, neovascularization	[37]
ADSCs from human, xenogeneic, 3 × 10^5	GG-HA spongy hydrogel	Vascularization hydrogels	Cells were seeded onto the top of spongy-like hydrogel sheets	STZ-induced diabetic mice	9 mm, dorsal	4 wk	Accelerated excisional skin wound healing; induced the healing phase switch from the inflammatory to the proliferative phase; presented a thicker epidermis with a high number of proliferative keratinocytes in the basal layer; increased the number of intraepidermal nerve fibers in the regenerated epidermis	[41]
BMSCs from rabbits, allogenic, 1 × 10^6	SNAP-loaded chitosan-PVA hydrogel	Vascularization hydrogels	Cells were intradermally injected and topically covered with hydrogel sheets	Alloxan monohydrate induced diabetic rabbits	20 mm, dorsal	14 d	Augmented the wound closure, decreased inflammation, and upregulated expression of CD31, VEGF and TGFβ-1; promoted angiogenesis by forming new capillaries and improving the microvascular and vessel maturation; showed an abundant expression of collagen type I on day 14	[44]
ADSCs from human, xenogeneic, 5 × 10^5	Curcumin-incorporated 3D bioprinting GelMA hydrogel	Antioxidant hydrogels	Cells were encapsulated into hydrogel sheets	STZ-induced diabetic nude mice	15 mm, dorsal	21 d	Promoted wound healing; improved hADSCs apoptosis and increased the amount of collagen	[46]
ADSCs from human, xenogeneic, 2.5 × 10^5	hDAM hydrogel	Intact ECM-derived hydrogels from living tissues	Cells were suspended in the *in situ* forming	KK/Upj-Ay/J mice (diabetic mice)	8 mm, dorsal	14 d	Accelerated wound closure and improved skin architecture regeneration,	[62]

			hydrogels				including better restoration of cutaneous appendages, increase of dermis thickness, and augmenting neovascularization	
UMSCs from human, xenogeneic, 5 × 10^6	GelMA/Chi-C hydrogel	Vascularization hydrogels	Cells were mixed with the *in situ* forming hydrogels	Diabetic mice (db/db)	8 mm, dorsal	14 d	Promoted the wound healing process by inhibiting protein expression of TNF-α and IL-1β to decrease inflammation. Accelerated angiogenesis and re-epithelialization, promoted collagen deposition, and induced regeneration of skin appendages such as hair follicles	[63]
PDSCs from human, xenogeneic, 1 × 10^6	Chitosan/collagen/β-GP hydrogel	Thermosensitive and pH-responsive hydrogels	3D spheroids were encapsulated in the *in situ* forming hydrogels	Diabetic mice (db/db)	7 mm, dorsal	3 wk	Accelerated wound closure by enhancing angiogenesis and paracrine effects. The hydrogel provided an environment favorable for the attachment and proliferation of encapsulated hPDSCs, accelerating cell proliferation and paracrine factor secretion	[67]
ADSCs from rats, allogenic, 5 × 10^5	Gelatin hydrogel	Adaptive hydrogel microspheres with degradation rates well-matched to tissue regeneration	Hydrogel microspheres	STZ-induced diabetic rats	8 mm, dorsal	14 d	Significantly accelerated wound healing by promoting M2 macrophage polarization, collagen deposition, angiogenesis associated with peripheral nerve recovery, and hair follicle formation. The microspheres well embedded in the tissue, exhibited good biocompatibility and adaptive biodegradation rates	[77]
BMSCs from human, xenogeneic, 5 × 10^5	PEGDA hydrogel	Bioinert synthetic hydrogels	Cells were encapsulated into hydrogel sheets	Genetically diabetic mice (BKS.Cg-m +/+Leprdb/J)	1 cm × 1 cm[1], dorsal	14 d	Accelerated wound healing; the co-encapsulation of hBMSCs and insulin secreting cells resulted in healing wounds without scab or scar	[79]
ADSCs from human, xenogeneic, 3 × 10^5	PEG-gelatin hydrogel	Vascularization hydrogels	Cells were mixed with the *in situ* forming hydrogels	Diabetic mice (db/db)	6 mm, dorsal	15 d	Significantly accelerated wound closure; the encapsulated cells attached and diffused well inside the hydrogel, improving cell retention *in vivo*; reduced inflammatory cell infiltration and enhanced neovascularization	[80]

[1]Wound size (side length × side length).

3D: Three dimensional; ADSCs: Adipose-derived stem cells; β-GP: β-glycerophosphate; BMSCs: Bone marrow-derived mesenchymal stem cells; Chi-C:

Chitosan-catechol; ECM: Extracellular matrix; GelMA: Gelatin methacryloyl; GG-HA: Gellan gum-hyaluronic acid; HA-ALD: Hyaluronic acid-aldehyde; hADSCs: Human adipose-derived stem cells; hBMSCs: Human bone marrow-derived mesenchymal stem cells; hDAM: Human decellularized adipose tissue matrix; hPDSCs: Human placenta-derived mesenchymal stem cells; N-chitosan: N-carboxyethyl chitosan; PDSCs: Placenta-derived mesenchymal stem cells; PEG: Poly(ethylene glycol); PEGDA: Polyethylene glycol diacrylate; PVA: Polyvinyl alcohol; rBMSCs: Rat bone marrow-derived mesenchymal stem cells; SNAP: S-nitroso-N-acetyl-penicillamine; STZ: Streptozotocin; UMSCs: Umbilical cord-derived mesenchymal stem cells; VEGF: Vascular endothelial growth factor.

FOOTNOTES

Author contributions: Li Y designed the research study; Huang JN and Cao H searched the literature and drafted the initial manuscript; Liang KY and Cui LP provided further revision; all authors have read and approved the final version of the manuscript.

Supported by the Shenzhen Basic Research Project, No. JCYJ20190807155805818; and the Foundation of Guangdong Provincial Key Laboratory of Sensor Technology and Biomedical Instrument, No. 2020B1212060077.

Conflict-of-interest statement: There are no conflicts of interest to report.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: Filipodia
P-Editor: Chen YL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 962-971

DOI: 10.4239/wjd.v13.i11.962

ISSN 1948-9358 (online)

MINIREVIEWS

Role of defensins in diabetic wound healing

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Specialty type: Engineering, biomedical

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Dziegielewska-Gesiak S, Poland; Salim A, Pakistan

Received: September 14, 2022
Peer-review started: September 14, 2022
First decision: October 5, 2022
Revised: October 22, 2022
Accepted: October 31, 2022
Article in press: October 31, 2022
Published online: November 15, 2022

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Abstract

The adverse consequences resulting from diabetes are often presented as severe complications. Diabetic wounds are one of the most commonly occurring complications in diabetes, and the control and treatment of this is costly. Due to a series of pathophysiological mechanisms, diabetic wounds remain in the inflammatory phase for a prolonged period of time, and face difficulty in entering the proliferative phase, thus leading to chronic non-healing wounds. The current consensus on the treatment of diabetic wounds is through multidisciplinary comprehensive management, however, standard wound treatment methods are still limited and therefore, more effective methods are required. In recent years, defensins have been found to play diverse roles in a variety of diseases; however, the molecular mechanisms underlying these activities are still largely unknown. Defensins can be constitutively or inductively produced in the skin, therefore, their local distribution is affected by the microenvironment of these diabetic wounds. Current evidence suggests that defensins are involved in the diabetic wound pathogenesis, and can potentially promote the early completion of each stage, thus making research on defensins a promising area for developing novel treatments for diabetic wounds. In this review, we describe the complex function of human defensins in the development of diabetic wounds, and suggest potential therapeutic benefits.

Key Words: Defensin; Diabetic wound; Wound healing; Inflammation; Re-epithelialization; Tissue regeneration

Core Tip: Although previous studies have suggested that defensins have a function in the promotion of wound healing, their mechanism is still unclear. In this review, we discuss the potential role of various defensins in refractory diabetic wounds and their properties, including immunoregulation, promotion of re-epithelialization, collagen deposition, vascular regeneration, and neurological recovery, as well as antimicrobial activity.

Citation: Tan ZX, Tao R, Li SC, Shen BZ, Meng LX, Zhu ZY. Role of defensins in diabetic wound healing. *World J Diabetes* 2022; 13(11): 962-971
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/962.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.962

INTRODUCTION

Diabetes was estimated to affect over 536.6 million people worldwide in 2021, and an increase in prevalence occurs at a faster rate among middle-income countries[1]. Diabetes mellitus gradually causes a series of complications, such as neuropathy, retinopathy, nephropathy, cardiovascular diseases, and diabetic wounds, as it develops. Due to lesions in the small blood vessels and peripheral nerves that are particularly prominent in the feet, diabetic wounds are usually presented as diabetic foot ulcers (DFUs), and are characterized by a delayed tissue growth and increased susceptibility to infection. Patients with DFUs have an increased risk of lower limb amputation, which has poor short-term prognosis associated with high mortality[2]. Statistically, the five-year mortality and direct costs of care for patients with DFUs have been comparable to that of cancer[3]. The large patient population and medical expenditure urgently requires an effective treatment method.

The mechanisms for diabetic wound development involve multifactorial etiologies, including a hyperglycemic microenvironment, abnormal host immune resistance, and neuropathy (Figure 1)[4]. These mechanisms influence each other, instead of occurring independently, causing irreversible diabetic complications. The local damage to vessels and nerves, reduced growth factors expression, and lower collagen accumulation contribute to repeated outbreaks and the protracted course of diabetic wounds, leading to further infections. Although current treatments, including glycemic control, anti-infective treatment, and advanced dressing application, promote wound healing by regulating the local microenvironment, they also have disadvantages, such as protracted treatment periods, high costs, and occasionally inefficient results[5]. Hence, studying methods that promote diabetic wound healing is ongoing.

With concern about antibiotic resistance growing more prominent, antimicrobial peptides (AMPs) have garnered attention as a new method of antibacterial therapy, including development of different formulation strategies for effective delivery to wounds, including AMPs loaded in nanoparticles, hydrogels, creams, gels, *etc*. As a representative AMP, the defensins properties are gradually being researched (Table 1)[6-23]. Human defensins are divided into α-defensins and β-defensins[23]. Human α-defensins mainly occur in neutrophils (human neutrophil peptide1-4, HNP1-4) or small intestinal Paneth cells (human defensin5-6, HD5-6). More extensively, 31 human β-defensins (HBDs) have been described, and HBD1-4 is most widely-studied[24]. Reportedly, the direct primary role of defensins is controlling microbial infections by killing bacteria and modulating the immune system. Moreover, defensins play different roles in different environments within the body, such as infected wounds, malignancy, atherosclerosis, pulmonary fibrosis, *etc*. In this review, we focused on investigating their mechanism of action in wound healing, especially chronic diabetic wounds.

Although little is known about defensins involvement in diabetic wounds, existing studies indicate that they play potential roles in complex pathophysiological changes of diabetic wounds[25,26]. This review summarizes and analyzes known experimental data about the role of defensins in diabetic wound healing, particularly for inflammation, cell proliferation and migration, regeneration of blood vessels and nerves, and antibacterial activities. Research articles on the role of defensins in diabetic wounds, published between inception and September 10, 2022, were collected from various search engines, such as PubMed, Google Scholar, Web of Science, and Science Direct using the following keywords: AMPs, defensins, host defense peptides, diabetic, refractory, and chronic wounds, wound healing, *etc*. Identified studies and relevant citations within these studies were reviewed.

MULTIFACTORIAL MECHANISM OF DEFENSINS DURING WOUND HEALING

The response to tissue injury involves multiple cellular and extracellular events, including inflammation, re-epithelialization, and angiogenesis, followed by fibroplasia with collagen synthesis, and tissue remodeling. Defensins may be a multifactorial modulator in the management of this process,

Table 1 Defensins play multiple roles in different diseases

Defensin		Main cellular source	Action	
α-defensin	HNP1	Neutrophils; monocytes; macrophages; natural killer cells	Increase the healing rate of MRSA-infected wounds[6]; promote hemostasis[7]; r/affect the cardiovascular system[8]; inhibit thrombus formation[9]	Anti-infection and immunoregulation [23]
	HNP2-3		Anti-tumor activity[10]	
	HNP4	Neutrophils	Characterize benign and malignant salivary gland tumors[11]	
	HD5-6	Intestinal Paneth cells	Reverse dyslipidemia and improve glucoregulatory capacity [12]; anti-tumor ability[13]; amyloid inhibitor[14]	
β-defensin	HBD1	Epithelial cells; monocytes; macrophages	Anti-tumor activity[15]; potentiate osteoclastogenesis[16]	Induce the secretion of angiogenin[54]; anti-infection and immunoregulation [23]
	HBD2		Accelerate wound healing[17]; Oncolytic activity[18]; reduce alcoholic liver injury[19]	
	HBD3		Accelerate wound healing[20]; induce IL-8 release and apoptosis in airway smooth muscle cells[21]	
	HBD4	Epithelial cells	Stimulate/suppress cancer cell proliferation and viability[22]	

HNP: Human neutrophil peptide; HBD: Human β-defensins.

which interferes in diabetic wounds (Table 2, Figure 2).

Defensins triggered by inflammation

The first phase of wound healing is the inflammatory phase, characterized by platelet aggregation and leukocytes migration, including neutrophils and macrophages that secrete defensins and consequently clear the wound area[27]. Studies suggest defensins promote recruitment and accumulation of leukocytes at inflammatory sites, and simultaneously release a series of chemokines[28,29]. In diabetic wounds, the number of neutrophils increases abnormally and macrophage polarization is suppressed, leading to an excessive inflammatory expression[30,31]. As defensins are released in response to inflammation from neutrophils and macrophages, which act as a signal to instigate recruitment of immune cells, a positive-feedback loop is created. HBDs can reportedly participate in degranulation of mast cells and induce secretion of proinflammatory factors by keratinocytes *via* the p38 and ERK1/2 MAPK pathways activation[32,33]. Through the same action sites, HNPs produce vasoactive by-products in endothelial cells *via* ROS-dependent mechanisms, and stimulate the increased expression of IL-6 and IL-8 by activating p42/44 MAPK pathways[34,35].

In contrast, studies investigating associations between defensins and inflammatory mediators exhibited controversial results. HBDs have demonstrated an immunosuppressive effect by down-regulating the TIR, TRAF-6, and NF-κB of TLR signaling pathways[36]. Moreover, HBDs contribute to their anti-inflammatory ability by inducing M2 macrophage differentiation[37]. Previous experiments established that HBDs can be beneficial in inflammatory diseases, such as periodontitis, considering its anti-inflammatory properties[38]. A study on HNPs from dying neutrophils exhibited an immunosuppressive effect of the α-defensins that inhibited macrophage stimulation[39]. The HNP1 "bipolar effect" represents the reduction of inflammatory responses with a physiological dose, enhanced expression of inflammatory factors with a high dose, and significant reductions in cell viability and interleukin-10 expression with increased concentration levels[40]. Overall, defensins perform different functions under different conditions, including concentration levels[40]. Defensins are often used as disease-related markers as dysregulation of their levels is caused by immune system disorders and effectors produced themselves or through associated cells[41]. However, the causal relationship and sequence of cascades remain unclear. Several studies emphasized the relationship between delayed wound healing and uncontrolled inflammatory responses, and defensins as efficient adjustors playing a regulatory role in the process.

Defensins promote skin reconstruction

Failure to re-epithelialize is one of the most significant indicators for chronic wounds. Re-epithelialization is achieved through keratinocyte migration, proliferation, and differentiation. The HNP1, HBD2, HBD3, and HBD4 can reportedly induce proliferation and migration of keratinocytes, which can consequently secrete HBDs, thereby promoting reconstruction of the cellular barrier to accelerate wound healing[42,43]. Subsequent studies reported that HBD3 enhances phosphorylation of the FGFR1/JAK2/STAT3 pathways to promote keratinocyte proliferation and migration[20]. HBD1 potentially acts as a relevant transcription factor by protecting keratinocytes from apoptosis during epithelial reorganization[44]. In other words, defensins have properties that promote wound epithelial-

Table 2 Defensins play a potential role in wound healing

Stage	Defensin	Activation
Inflammation	HNP1-2, HBD1-3[28]	Recruitment of leukocytes
	HNP1-4[29]	Secretion of inflammatory cytokines like IL-8
	HBD2-4[32]	Activation of the p38 and ERK1/2 MAPK pathways
	HNP1, HBD2[35]	Activation of the p42/44 MAPK pathways
	HBD2, HBD3[36]	Down-regulate the TIR, TRAF-6, NF-κB of TLR signaling pathways
	HBD3[37]	Induce M2 macrophage differentiation
Re-epithelialization	HNP1[43], HBD2-3[42]	Induce keratinocyte migration and proliferation
	HBD1[44]	Protect keratinocytes from apoptosis
Collagen synthesis	HNP1[45]	Enhance extracellular matrix deposition
	HBD3[20,37]	Increase the expression of MMP-2, and down-regulate the expression of MMP-9
Fibroplasia	HNP1[45], HBD2[46]	Promote the proliferation and activation of fibroblasts
Angiogenesis	HNP1[51], HBD2[52], HBD3[53]	Induce VEGF
	HBD1-4[54]	Induce angiogenin
	HNPs[55]	Inhibit adhesion and migration of endothelial cell
Nerve reconstruction	HNP1[40]	Promote the recovery of neurological function
	HBD3[57]	Modulate the expression of nerve elongation factors
Antimicrobial activity	HNP1-4, HBD1-4[61]	Exhibit a broad range of antimicrobial properties
	HBD2[60]	Reduce biofilm formation
	HNP1-3[62]	Neutralize bacterial toxins
	HNP1, HBD1, HBD3[63]	Show synergy of action with antibiotics

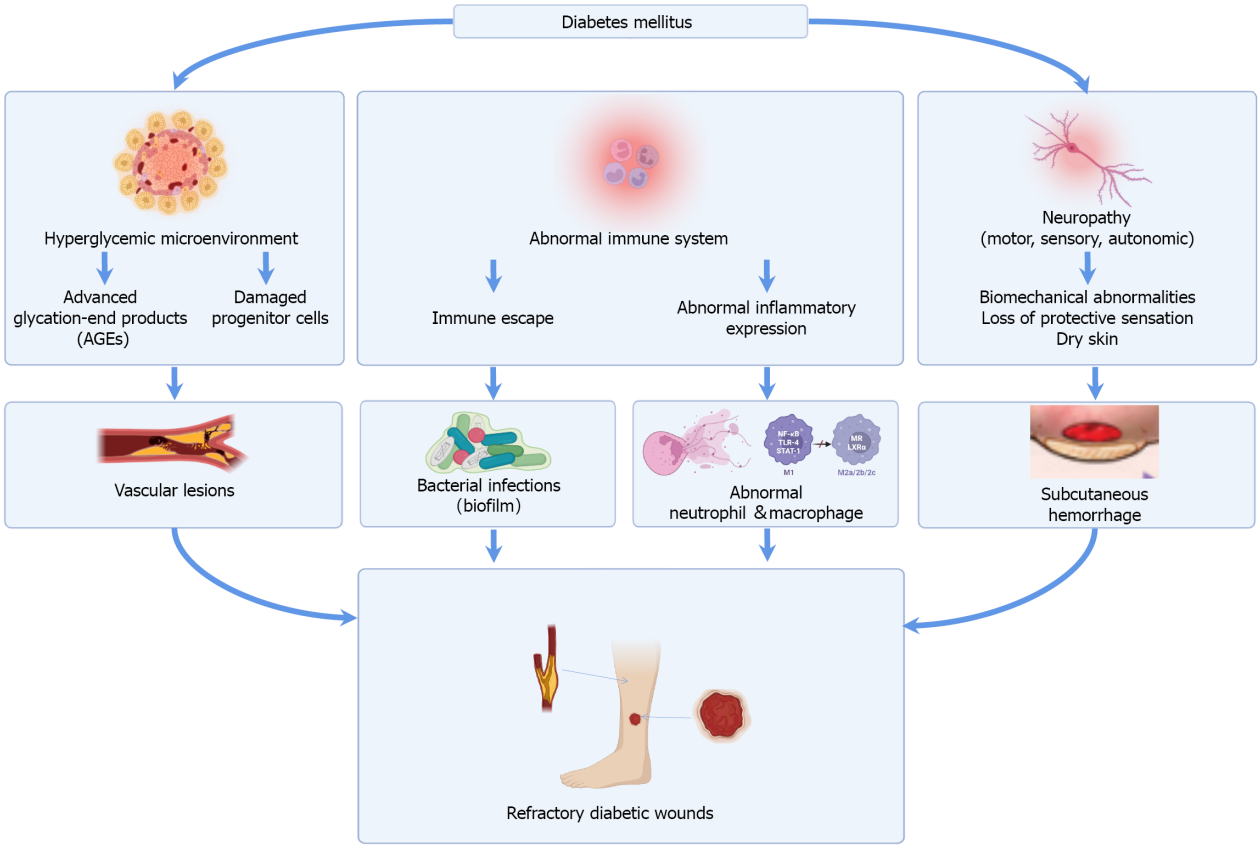
HNP: Human neutrophil peptide; HBD: Human β-defensins; MMP: Matrix metalloproteinase.

ization by affecting keratinocyte activity, and thus facilitating early wound closure.

Furthermore, defensins seemingly play an important role in fibroblasts and collagen matrix accumulation, which is essential for dermal reconstitution. HNP1 can reportedly promote proliferation and activation of fibroblasts more effectively than HBD2 at the same concentration, and the increased collagen gene expression can only be observed by its stimulation[45]. A study also proved that HBDs indirectly stimulate fibroblast migration by activating protein kinase C[46]. High levels of pro-inflammatory cytokines and inflammatory chemokines in diabetic wounds lead to an increased production of matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, thereby inhibiting extracellular matrix formation and dermis reconstruction[47,48]. Studies have suggested that the use of an inhibitor for MMP-2 and MMP-9 accelerates wound healing in diabetic mice by maintaining the balance between systematic inflammation and cytokine biosynthesis[49]. HBD3 may potentially reverse the pathological condition as they have shown an inhibitory effect on MMP-9, which may result from cytotoxicity for dendritic cells in high concentrations[50]. Instead, HBD3 reportedly increases the expression of MMP-2, which is essential for angiogenesis and prolonged matrix remodeling[20]. To explain these contradictory findings, further clarification and a comprehensive analysis on the mechanism of wound healing is necessary, as well as verification through specific experiments.

Defensins involved in regeneration of blood vessels and nerves

Angiogenesis is a vital physiological process in wound healing and largely regulated by growth factors, specifically vascular endothelial growth factor (VEGF) and angiogenin. HNP1, HBD2, and HBD3 was proven to bind to cell surface receptor proteins, thus promoting VEGF expression and improvement of vascularization[51-53]. The novel role of HBDs in angiogenesis was also identified, revealing that HBD1-4 increases secretion of angiogenin dose-dependently[54]. However, the opposing actions can be described as a consequence of on-site recruitment of distinct subpopulations from circulation. HNPs can reportedly inhibit adhesion and migration of endothelial cells, and block VEGF-induced endothelial cell proliferation and capillary formation upon inflammatory stimulation[55]. These studies have shed a light on the mechanistic complexity of HNPs angiogenesis.



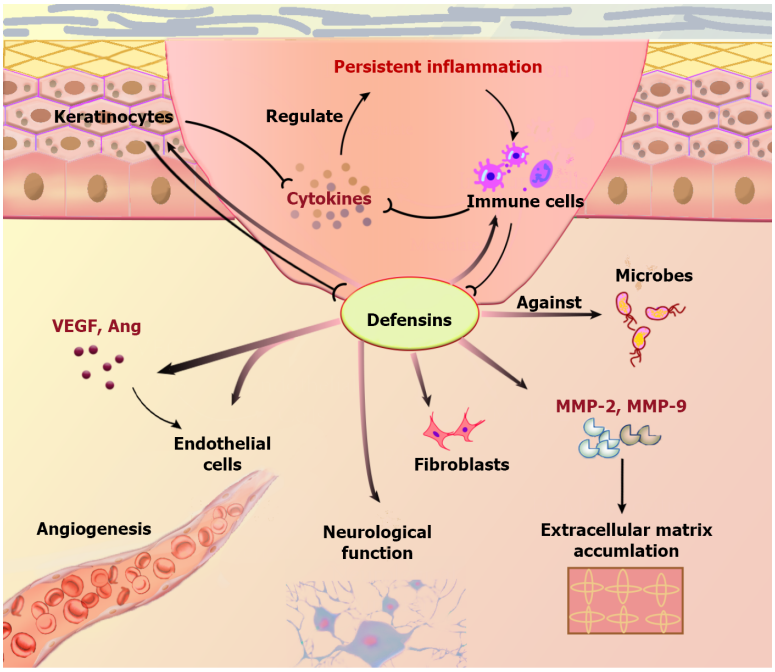
DOI: 10.4239/wjd.v13.i11.962

Figure 1 Mechanism of refractory diabetic wounds. The mainstream views include: Hyperglycemic microenvironment, abnormal immune system and neuropathy. Hyperglycemic microenvironment results in the complex formation of advanced glycation end products (AGEs) and cytokines, as well as circulating progenitor cell dysfunction. AGEs can significantly inhibit the proliferation of endothelial cells and alter the structure of collagen and elastin in the vascular wall, causing microvascular injury in the wound. The hallmarks of abnormal immune system are polymorphonuclear cell dysfunction, late neutrophil infiltration and suppressed macrophage polarization. As a result, diabetic wound healing is delayed and susceptible to bacterial infections and even biofilm formation. Neuropathy is occasion of subcutaneous hemorrhage, ultimately leads to skin breakdown.

Neuropathy caused by diabetes is the influencing factor for subcutaneous hemorrhage underneath the callus formation, ultimately leading to skin breakdown[56]. Studies have proven that HNP1 administration can promote recovery of neurological function following sciatic nerve injury[40]. Additionally, HBD3 modulates the expression of nerve elongation factors that are involved in epidermal hyperinnervation and hypersensitivity to warm sensations[57]. As a result, application of defensins can help prevent delayed treatment due to peripheral neuropathy and difficulty in mastering wound conditions in patients with diabetic wounds.

Defensins exhibit antimicrobial activity

Healing of refractory diabetic wounds is often associated with susceptibility to bacterial infections and formation of biofilms[58]. As a class of small cationic molecule peptides with broad-spectrum antimicrobial activity, defensins are produced to eliminate invading pathogens during the initial stages of wound formation[59]. While the important role of the pore-formation mechanism has been recognized in many studies, other mechanisms, such as disruption of cell wall synthesis, metabolic activity, ATP and nucleic acid synthesis, and amino acid uptake, have also been proposed in recent years[60]. HNPs and HBDs both exhibit a strong tendency to eliminate various pathogens, including *Staphylococcus aureus* and *Escherichia coli*, which often invades chronic wounds[61]. Specifically, HBD2 exhibits biofilm inhibitory activity by inducing structural changes that interfere with the biofilm precursor's transport into the extracellular space[60]. Additionally, HNPs were proven to protect leukocytes from neutralization by gram-positive pathogenic bacterial toxins[62]. Furthermore, they can potentially avoid the emergence of resistance when implemented with other antimicrobial therapies[63]. Defensins are not only more effective against drug-resistant bacteria, as compared to antibiotics, but can also preserve the resident bacteria, despite the lack of target specificity as an intractable problem preventing their use as a therapeutic drug[64].



DOI: 10.4239/wjd.v13.i11.962

Figure 2 Role of defensins in diabetic wound healing. VEGF: Vascular endothelial growth factor; Ang: Angiogenin.

CONCLUSION

Although there is ambiguity regarding its exact role, refractory healing of diabetic wounds is speculated result from interactions between multiple pathophysiological changes in the microenvironment of hyperglycemic and persistent inflammation. This affects immune cell function and composition of defensins at the wound site. In human skin, HBD1 is constitutively expressed in epithelial cells, while inducible HNP1-4 by neutrophils and HBD2-3 by keratinocytes mainly[65]. It was obtained through a biopsy that HBD2-4 were overexpressed in the border area of DFUs[25]. Studies generally agree that inadequate HBD expression is associated with poor wound healing, and many methods that promote diabetic wound healing are seemingly carried out by promoting defensins expression[66,67]. In diabetic wounds, higher HNP1, HNP3, and HNP4 expressions are more common in the central part than in the marginal areas, thus causing a significant increase in IL-8 expression under the influence of advanced glycation end products (AGEs)[29].

Defensins affect the expression and secretion of cytokines, cell proliferation, migration, and apoptosis, and are also involved in all stages of wound healing. Contrary to its proven activity in fighting pathogens and promoting tissue reconstruction, the role of defensins in inflammation and vascularization remains unclear. This discrepancy could be due to pro-inflammatory and anti-inflammatory properties being attributed to HBDs at lower concentrations[28,29], compared to antibacterial effects at higher concentrations exhibited in different experiments[48]. Thus, effects of defensins may vary depending on concentration. Furthermore, HNP1 and HBD3 exhibit increased cytotoxic effects with the increased concentration, which can also be related to a greater hydrophobicity[43,68]. Therefore, changing the local distribution or structure of defensins can have beneficial effects and prevent toxic side effects.

Studying every type of defensins within a single experiment is difficult. Additionally, certain defensins can exhibit different or contradictory effects within the same environment due to differences in experimental complexity and aims of the experiment. These factors create a huge obstacle in horizontal comparison among similar experiments. Cytotoxicity caused by defensins is difficult to assess, which indicates that topical application may be more appropriate than the systemic administration. Considering the unstable biochemical properties of defensins, topical application alone may be insufficient. To overcome this limitation, researchers are studying biological dressings as an alternative; however, formulation of an ideal material has not yet been achieved. However, animal studies on defensins exhibit improved healing outcomes, and display stable effects through application of new materials or genetic engineering methods[17,20,69]. These findings present defensins as a promising therapeutic approach owing to modern techniques, such as development of new materials to efficiently load active factors and novel protein sequences to highlight their beneficial effects.

Defensins regulates chronic inflammation, tissue regeneration, angiogenesis, and nerve recovery, as well as antimicrobial properties; therefore, they are a promising treatment for diabetic wounds. There is an urgent need to find the appropriate dosing regimens and develop new biological dressing altern-

atives to incorporate active factors. Hence, further preclinical investigations are necessary to understand extensive molecular mechanisms of defensins in the treatment of diabetic wounds, and consequently determine suitable therapeutic strategies.

FOOTNOTES

Author contributions: Tan ZX and Tao R wrote the manuscript and proposed research subtopics; Shen BZ was responsible for navigating the literature, sharing the relevant studies, and drawing the tables included in this review; Meng LX and Li SC drew the figures in the manuscript, formatted citations and compiled references, verified spelling, punctuation, and grammatical errors; Zhu ZY revised and formatted the body of the manuscript, and coordinated the whole work.

Supported by the Scientific Research Project of Hubei Health Committee, No. WJ2021F106.

Conflict-of-interest statement: Authors declare no conflicts of interests for this article.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 972-985

DOI: 10.4239/wjd.v13.i11.972

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Dietary N$^{\varepsilon}$-(carboxymethyl) lysine affects cardiac glucose metabolism and myocardial remodeling in mice

Zhong-Qun Wang, Zhen Sun

Specialty type: Cell biology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Jovandaric MZ, Serbia; Mai S, Italy

Received: August 16, 2022
Peer-review started: August 16, 2022
First decision: September 4, 2022
Revised: September 15, 2022
Accepted: October 11, 2022
Article in press: October 11, 2022
Published online: November 15, 2022

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Abstract

BACKGROUND

Myocardial remodeling is a key factor in the progression of cardiovascular disease to the end stage. In addition to myocardial infarction or stress overload, dietary factors have recently been considered associated with myocardial remodeling. N$^{\varepsilon}$-(carboxymethyl)lysine (CML) is a representative foodborne toxic product, which can be ingested *via* daily diet. Therefore, there is a marked need to explore the effects of dietary CML on the myocardium.

AIM

To explore the effects of dietary CML (dCML) on the heart.

METHODS

C57 BL/6 mice were divided into a control group and a dCML group. The control group and the dCML group were respectively fed a normal diet or diet supplemented with CML for 20 wk. Body weight and blood glucose were recorded every 4 wk. ^{18}F-fluorodeoxyglucose (FDG) was used to trace the glucose uptake in mouse myocardium, followed by visualizing with micro-positron emission tomography (PET). Myocardial remodeling and glucose metabolism were also detected. *In vitro,* H9C2 cardiomyocytes were added to exogenous CML and cultured for 24 h. The effects of exogenous CML on glucose metabolism, collagen I expression, hypertrophy, and apoptosis of cardiomyocytes were analyzed.

RESULTS

Our results suggest that the levels of fasting blood glucose, fasting insulin, and serum CML were significantly increased after 20 wk of dCML. Micro-PET showed that ^{18}F-FDG accumulated more in the myocardium of the dCML group than in the control group. Histological staining revealed that dCML could lead to myocardial fibrosis and hypertrophy. The indexes of myocardial fibrosis, apoptosis, and hypertrophy were also increased in the dCML group, whereas the activities of glucose metabolism-related pathways and citrate synthase (CS) were

significantly inhibited. In cardiomyocytes, collagen I expression and cellular size were significantly increased after the addition of exogenous CML. CML significantly promoted cellular hypertrophy and apoptosis, while pathways involved in glucose metabolism and level of *Cs* mRNA were significantly inhibited.

CONCLUSION

This study reveals that dCML alters myocardial glucose metabolism and promotes myocardial remodeling.

Key Words: Diet; Myocardial remodeling; Glucose metabolism; Nε-(carboxymethyl)lysine; C57 BL/6 mice

Core Tip: N^{ε}-(carboxymethyl)lysine (CML) exists in daily diet and is harmful to health. We established *in vitro* and *in vivo* models to investigate the effects of dietary CML (dCML) on the heart. We found that long-term dCML induced insulin resistance and elevated serum CML level. ^{18}F-fluorodeoxyglucose imaging indicated that dCML promoted myocardial glucose uptake, but the glucose metabolism was disrupted. Myocardial fibrosis, apoptosis, and hypertrophy were significantly enhanced by dCML. In the cell model, CML supplementation promoted cardiomyocyte apoptosis, cellular hypertrophy, and collagen I expression, and also inhibited pathways involved in glucose metabolism.

Citation: Wang ZQ, Sun Z. Dietary N^{ε}-(carboxymethyl) lysine affects cardiac glucose metabolism and myocardial remodeling in mice. *World J Diabetes* 2022; 13(11): 972-985
URL: https://www.wjgnet.com/1948-9358/full/v13/i11/972.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i11.972

INTRODUCTION

Cardiovascular disease is the leading cause of mortality worldwide[1]. Myocardial remodeling can lead to decreased cardiac function, which is an important factor in increasing mortality due to cardiovascular disease[2]. Cardiac remodeling is characterized by cardiomyocyte hypertrophy, apoptosis, fibrosis, and increased fibrocollagen deposition[3]. Short-term compensatory remodeling increases cardiac contractility, whereas long-term sustained pathological remodeling leads to a decline in cardiac function or even heart failure[4]. Cardiac remodeling can be caused by myocardial infarction, stress overload, inflammatory cardiomyopathy, idiopathic dilated cardiomyopathy, or diabetes[5]. Although these causes differ, they share similar mechanisms such as oxidative stress, endoplasmic reticulum stress, and inflammatory response[6]. Attempts have been made to improve myocardial remodeling with drugs or other interventions, albeit with unsatisfactory results[7].

Recent studies have shown that in addition to diseases such as diabetes and coronary heart disease, foodborne factors are also associated with myocardial remodeling[8]. Nakamura *et al*[9] found that carbohydrate content in the diet could affect myocardial remodeling. Zeng *et al*[10] reported that a high-fat diet promoted myocardial remodeling. Therefore, it is important to identify the key pathogenic components in the diet for the prevention and treatment of myocardial remodeling.

Advanced glycation end products (AGEs) are a class of heterogeneous irreversible products formed by non-enzymatic reactions[11]. AGEs can accumulate in various tissues resulting in adverse health effects by increasing disease pathogenesis[12,13]. AGEs can accumulate *via* endogenous and exogenous mechanisms. Food is the main source of exogenous AGEs[14]. N^{ε}-(carboxymethyl)lysine (CML) is considered a representative of food-derived AGEs[15]. CML has been found in a variety of foods such as milk, bakery products, and coffee. Ahmed *et al*[16] reported that the concentration of CML is 877 ± 47 nM in pasteurized milk. Assar *et al*[17] reported that bread crust contains 46.1 mg/kg of CML. Ingestion of CML *via* routine diet is substantially higher than the level of CML in plasma and tissues[18]. Daily exposure to high levels of CML is a health risk for humans[19]. The dietary intake of CML is positively correlated with prevalent vertebral fractures[20]. Studies have also shown that foodborne CML can accelerate the progression of atherosclerosis, Alzheimer's disease, and other diseases[21]. Our previous studies reported that long-term exposure to CML leads to osteogenic differentiation of vascular smooth muscle cells and calcification in diabetic plaques[12]. Exogenous CML can lead to the continuous evolution of atherosclerotic plaques[22]. However, it is currently unknown whether CML intake affects myocardial remodeling.

Glucose metabolism is an important energy source for heart activities. Physiologically, cardiomyocytes uptake and transport glucose *via* the glucose transporter (Glut) family and obtain energy *via* aerobic glucose oxidation[23]. Under pathological conditions such as myocardial infarction, glucose metabolism is altered and glucose uptake is increased to counteract the decline in cardiac function[24]. Impaired glucose metabolism is an independent risk factor for the progression of heart failure[25]. However, whether foodborne factors have an impact on myocardial glucose metabolism needs further study.

In the present study, we hypothesized that dietary CML (dCML) can lead to myocardial glucose metabolism dysfunction and myocardial remodeling. We fed mice a CML-supplemented diet and used ^{18}F-fluorodeoxyglucose (FDG) to track the glucose uptake by the mouse myocardium *in vitro* and *in vivo*. Our study provides new insights into the relationship between dCML and myocardial remodeling.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Experimental Animal Use Ethics Committee of Jiangsu University and followed the ARRIVE guidelines. Ten-wk-old male C57 BL/6 mice (Cavens, ChangZhou, China) were stored in a light:dark (12:12) cycle environment at a temperature of 26 °C and humidity of 70%. Mice were randomly divided into two groups: control (Ctrl) group and dCML group (n = 25). Mice in the Ctrl group were freely administered a standard pelleted diet (XieTong, Nanjing, China). The dCML group received a daily standard pelleted diet mixed with CML (1 g/kg)[26]. The body weight and blood glucose of mice were measured every 4 wk. Blood glucose was detected with a glucose meter (Roche, Basel, Switzerland). After 20 wk of feeding, the fasting insulin levels of the mice were detected *via* the enzyme-linked immunoassay (ELISA) (Mercodia, Sweden), and the homeostatic model assessment insulin resistance (HOMA-IR) was calculated [HOMA-IR = glucose (mmol/L) × insulin (mIU/L)]/22.5. Then the mice were subjected to the oral glucose tolerance test (OGTT)[27]. Each mouse was fasted for 8 h prior to the tests. During fasting, mice had access to adequate water. The serum CML level in mice was detected *via* ELISA (Cloud-Clone, Wuhan, China). The operation steps followed the instructions. The citrate synthase (CS) in mouse tissues was detected with the Citrate Synthase Activity Detection Kit (Solarbio, Beijing, China). The mouse tissues were homogenized and centrifuged at 11000 g for 10 min. The protein concentration of supernatants was measured using the bicinchoninic acid assay. The samples were reacted with the detection reagent for 10 s or 2 min, and the absorbance at 412 nm was measured. Then the activity was calculated and expressed as nmol/min/mg protein.

Histology

Mice were euthanized with carbon dioxide (CO_2). Mouse hearts were isolated and the left ventricles were used for subsequent analysis. Masson's trichrome staining was performed to assess the degree of myocardial fibrosis in mice using an appropriate kit (Solarbio). Myocardial glycogen accumulation was measured using the Glycogen Periodic Acid Schiff (PAS) Stain Kit (Solarbio)[28]. The cytoplasm and nuclei were stained with hematoxylin and eosin (H&E) dye according to the manufacturer's instructions (Solarbio), followed by imaging using a microscope (Olympus, Tokyo, Japan). Areas testing positive with Masson's trichrome and PAS stains were measured using ImageJ software.

Positron emission tomography imaging

To assess glucose uptake in the mouse myocardium *in vivo*, ^{18}F-FDG was used to trace the glucose metabolism and visualized with micro-positron emission tomography (PET) (Inveon; Siemens, Munich, Germany)[29]. Mice were fasted for 12 h before scanning. The mice were weighed and anesthetized with isoflurane/oxygen mixture (15-20 mL/L). ^{18}F-FDG (7.4 MBq, 150 μL) was injected through the tail vein. Micro-PET scanning was performed 2 h after injection for 10 min. The scanned images were iteratively reconstructed with ordered set expectation maximization three-dimensional software. The average level of radioactive material uptake in the cardiac region was analyzed using ASIProVM software. The mean of standard uptake value (SUV_{mean}) was calculated according to the formula: SUV_{mean} = cardiac radioactive material uptake (μCi/g)/[total injected dose (μCi)/body weight (g)]. Similarly, the *in vitro* micro-PET scanning of mouse hearts was also performed[30]. Two hours after the injection of ^{18}F-FDG, the mice were euthanized with CO_2 and the hearts were isolated. The isolated heart was placed in a tube filled with ultrasound gel and scanned *via* micro-PET. The calculation of SUV_{mean} of isolated mouse hearts was the same as described above.

Cell culture

H9C2 cells (Procell, Wuhan, China) were cultured in Dulbecco's Modified Eagle Medium supplemented with 100 mL/L fetal bovine serum[31]. Cells were divided into a Ctrl group and a CML group, followed by seeding of 2 × 10^5 cells and addition of 10 mmol/L CML to the CML group, and incubated for 24 h.

Cell viability was detected with Cell Counting Kit-8 (C0037; Beyotime, Shanghai, China). All cells were cultured at 37 °C with 50 mL/L CO_2. Collagen I content in H9C2 cells was assessed *via* immunocytochemical staining. The SP Rabbit & Mouse HRP Kit (CoWin Century, Beijing, China) was used for immunocytochemical staining. Briefly, after fixing in 40 g/L paraformaldehyde for 30 min, the cell samples were washed with phosphate-buffered saline. The samples were incubated with 100 mL/L goat serum and 2.5 mL/L Triton X-100 at room temperature, followed by incubation with primary and secondary antibodies. The primary antibody used for the staining was: anti-collagen I (1:500, 14695-1-AP; Proteintech, Rosemont, IL, United States). To assess the cardiomyocyte area, cells were labeled with Phalloidin (P5282; Sigma-Aldrich, St. Louis, MO, United States). After fixing in 40 g/L paraformaldehyde, the cells were incubated with 2.5 mL/L Triton X-100 for 15 min, followed by labeling with Phalloidin (5 μmol/L) to analyze cell morphology. The nuclei were subsequently stained with DAPI. All stained images were acquired under a microscope (Olympus) and quantified with a computer-assisted image analysis system. Six high-resolution fields in each independent experiment were randomly selected and the area of at least 100 cells was calculated.

Western blotting

The experimental steps were performed as previously described[32]. Protein samples were prepared using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Beyotime). Proteins were transferred to PVDF membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After blocking in 50 g/L milk powder for 1 h, the membranes were incubated with suitable diluted primary antibodies overnight. The membranes were incubated with horseradish peroxidase-labeled secondary antibodies and imaged using a chemiluminescence system (Amersham Imager 600; GE Healthcare, Chicago, IL, United States). The acquired images were analyzed using ImageJ software. Primary antibodies used for western blotting were anti-CML (1:1000, ab125145; Abcam, Cambridge, United Kingdom), anti-collagen I (1:500, 14695-1-AP; Proteintech), anti-Glut-1 (1:2000, 21829-1-AP; Proteintech), anti-Glut-4 (1:2000, 66846-1-Ig; Proteintech), anti-Akt (1:5000, 10176-2-AP; Proteintech), anti-B-cell leukemia/lymphoma 2 (Bcl-2) (1:2000, 26593-1-AP; Proteintech), anti-Bcl-2-associated X, apoptosis regulator (BAX) (1:2000, 50599-2-Ig; Proteintech), anti-Akt (phospho-Ser473; 1:5000, 66444-1-Ig; Proteintech), anti-AMP-activated protein kinase (AMPK) (1:5000, 66536-1-Ig; Proteintech), anti-phospho-AMPK (phospho-Thr183 and Thr172; 1:5000, ab133448; Abcam) and anti-β-actin (1:2000, ET1702-67; HUABIO, Hangzhou, China).

Quantitative PCR

Total RNA from tissues/cells was obtained with the RNA-Quick Purification Kit (ES-RN001; YISHAN BIOTECH, Shanghai, China). The mRNA was reverse-transcribed into cDNA using a reverse transcriptase kit (R222; Vazyme Biotech, Nanjing, China)[33]. The SYBR qPCR Master Mix Kit (Q311, Vazyme Biotech) was used to detect the level of each gene. All experimental operations were carried out according to the manufacturer's instructions. The primer sequences are shown in Table 1.

Statistical analysis

All data were presented as the mean ± SD. The differences between the two groups were analyzed with the Student's *t*-test. Statistical significance was set at $P < 0.05$. All data were analyzed using SPSS 22.0 software.

RESULTS

Dietary CML affects glucose metabolism and insulin resistance in mice

After feeding the mice a diet supplemented with CML, we assessed their body weight and blood glucose every 4 wk until week 20. Body weight of the Ctrl group increased with feeding time, whereas the weight gain of the dCML group slowed down from the 8th wk of feeding (Figure 1A). There was no significant difference in body weight between the Ctrl group and the dCML group. From the 12th wk of feeding, the fasting blood glucose of mice in the dCML group was significantly higher than in the Ctrl group (Figure 1B). Further, we detected the glucose tolerance of mice. The OGTT test showed that the blood glucose AUC of the dCML group was significantly increased compared with the Ctrl group, suggesting impaired glucose tolerance of the dCML group (Figure 1C and D). There were significant differences in fasting insulin and HOMA-IR values between the two groups of mice. The fasting insulin and HOMA-IR levels of the dCML group were higher than those of the Ctrl group, suggesting insulin resistance in the dCML group (Figure 1E and F). Similarly, the serum CML level of mice in the dCML group was also significantly increased due to the consumption of CML, which was 2.6-fold higher than that in the Ctrl group (Figure 1G).

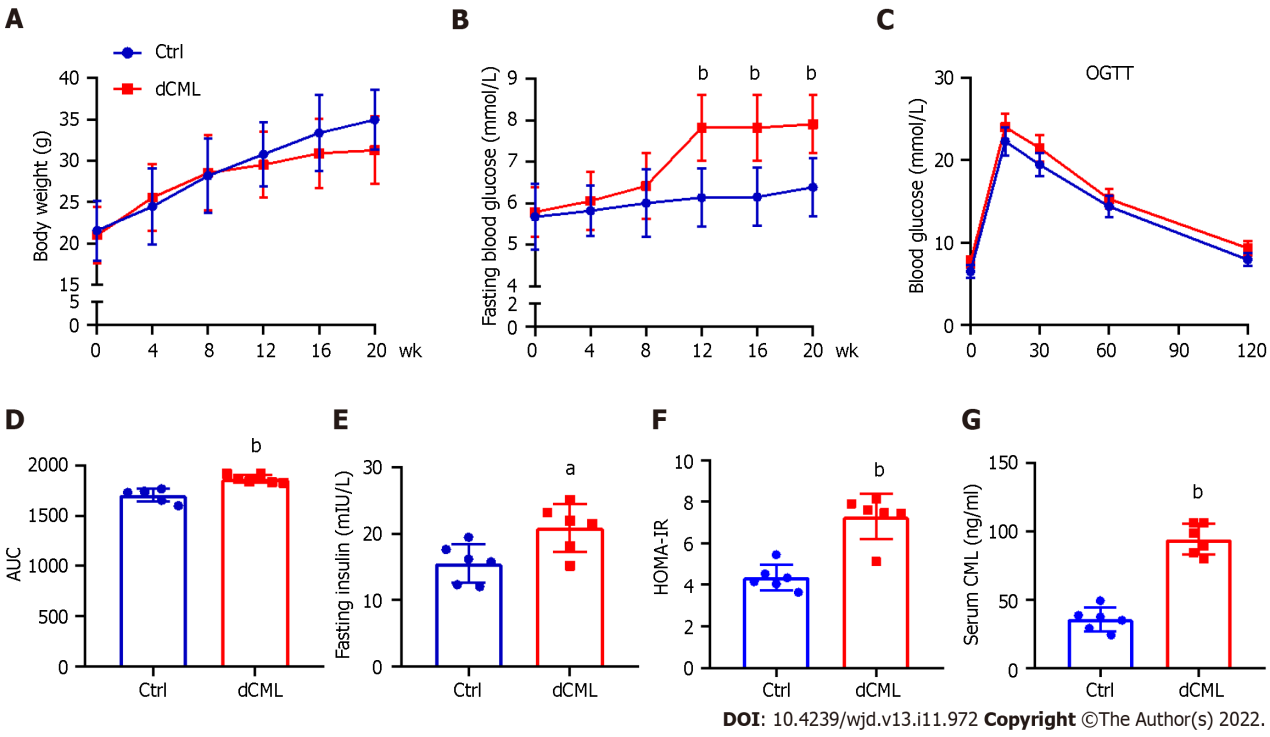
Dietary CML promotes glucose uptake in mouse myocardium

^{18}F-FDG is a glucose analog that can be used to track the glucose uptake in tissues and organs. Micro-

Table 1 Sequences of the primers used for quantitative PCR

Gene	Forward, 5'-3'	Reverse, 5'-3'
Bax (mouse)	GAACAGATCATGAAGACAGGG	CAGTTCATCTCCAATTCGCC
Bcl-2 (mouse)	AGGGGGAAACACCAGAATC	GGTAGCGACGAGAGAAGT
ANP (mouse)	CCTGTGTACAGTGCGGTGTC	CCTAGAAGCACTGCCGTCTC
Glut-1 (mouse)	ACGCCCCCCAGAAGGTTAT	GCGTGGTGAGTGTGGTGGAT
Glut-4 (mouse)	TTGCACACGGCTTCCGAACG	GATCTGCTGGAAACCCGACGG
β-actin (mouse)	TCTTGGGTATGGAATCCTGTG	ATCTCCTTCTGCATCCTGTCA
Bax (rat)	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG
Bcl-2 (rat)	AGTGGGATGCGGGAGATGTG	GGGGCCGTACAGTTCCACAA
ANP (rat)	CCGTATACAGTGCGGTGTCCAAC	TCATCGGTCTGCTCGCTCAGG
Glut-1 (rat)	GCCTGAGACCAGTTGAAAGCAC	CTGCTTAGGTAAAGTTACAGGAG
Glut-4 (rat)	AGGCACCCTCACTACCCTTT	AGCATAGCCCTTTTCCTTCC
Cs (rat)	GGAACACACTCAACTCGGGA	ACCCCACTGTGAGCATCTACG
β-actin (rat)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

ANP: Atrial natriuretic peptide; BAX: Bcl-2-associated X; Bcl-2: B-cell leukemia/lymphoma 2; Cs: Citrate synthase; Glut: Glucose transporter.



DOI: 10.4239/wjd.v13.i11.972

Figure 1 Dietary Nᵋ-(carboxymethyl)lysine increases blood glucose and induces insulin resistance in mice. A: Body weight of mice; B: Fasting blood glucose of mice; C: Oral glucose tolerance test (OGTT) test of mice; D: Area under the curve (AUC) of OGTT test; E: Fasting insulin of mice; F: Mouse homeostatic model assessment insulin resistance; G: Mouse serum Nᵋ-(carboxymethyl)lysine (CML) level. Ctrl: Control; dCML: Dietary CML; $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with the Ctrl group.

PET scanning was performed to detect the uptake of ^{18}F-FDG in the mouse myocardium. Compared with the Ctrl group, the myocardial SUV_{mean} of the dCML group was significantly increased (6.40 ± 0.70 *vs* 3.67 ± 0.60; $P < 0.01$) (Figure 2A and B). Then the mouse hearts were isolated and detected *via* micro-PET scanning *in vitro*. The SUV_{mean} of hearts in the dCML group was still significantly higher than that in the Ctrl group (0.71 ± 0.10 *vs* 0.41 ± 0.06; $P < 0.01$) (Figure 2C and D), suggesting that the glucose uptake of the myocardium was increased after supplementation with the CML diet.

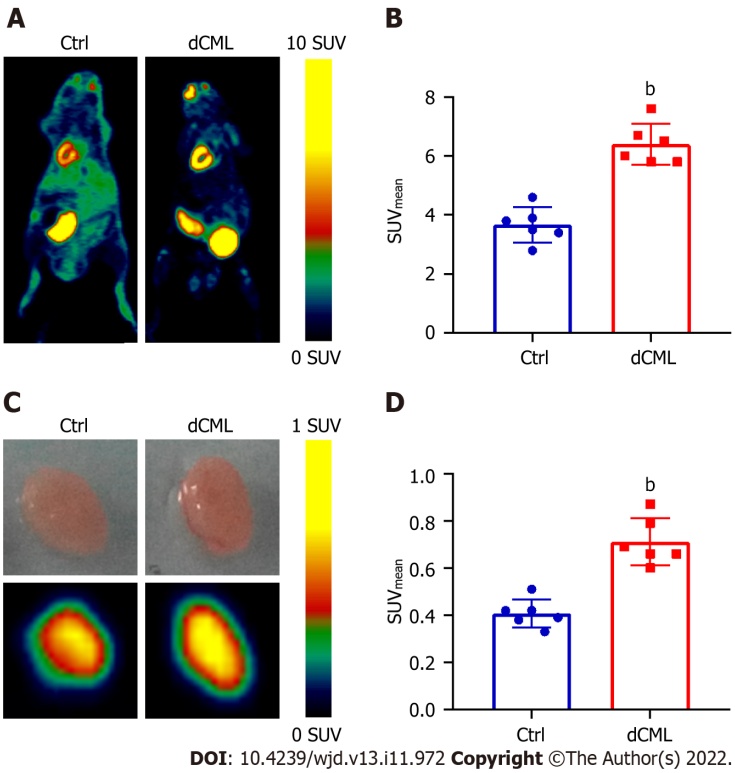


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Figure 2 Myocardial glucose uptake is increased after dietary N$^{\varepsilon}$-(carboxymethyl)lysine. A and B: Micro-positron emission tomography scanning of ^{18}F-fluorodeoxyglucose (FDG) accumulation in mouse myocardium; C and D: Uptake of ^{18}F-FDG by isolated mouse hearts of the control (Ctrl) group and dietary N$^{\varepsilon}$-(carboxymethyl)lysine (dCML) group. SUV: Standard uptake value. $n = 6$. $^{b}P < 0.01$, compared with the Ctrl group.

Dietary CML promotes myocardial remodeling in mice

Histopathological changes in the myocardium of mice were detected after 20 wk of dCML. Glycogen PAS staining showed a significant increase in the accumulation of glycogen in the myocardium of the dCML group, which was 3.7-fold higher than that of the Ctrl group, suggesting impaired glucose metabolism (Figure 3A and B). Masson staining indicated that myocardial fibrosis was aggravated in the dCML group (Figure 3A and C). H&E staining showed that the cell area in the myocardium of the dCML group was 1.32-fold higher than that of the Ctrl group (Figure 3A and D). The level of CML in the myocardium of the dCML group also significantly increased (Figure 3E and F). Protein levels of collagen I and the apoptosis regulator BAX were significantly upregulated, whereas the anti-apoptotic factor Bcl-2 was significantly inhibited (Figure 3E, G, H and I). The mRNA level of the cardiac hypertrophy indicator atrial natriuretic peptide (ANP) was significantly increased in the dCML group (Figure 3J). Compared with the Ctrl group, *Bax* mRNA was significantly upregulated, whereas *Bcl-2* was significantly downregulated in the dCML group, suggesting increased myocardial apoptosis in the dCML group (Figure 3K and L).

Dietary CML inhibits glucose metabolic signaling pathways in mouse myocardium

Glut-1 and Glut-4 play a key roles in myocardial glucose transport[34]. Glut-1 mRNA and protein levels were significantly increased in the mouse myocardium of the dCML group, whereas Glut-4 was significantly decreased (Figure 4A-E). Akt and AMPK signaling are key regulatory pathways in glucose metabolism[34,35]. Compared with the Ctrl group, the dCML group showed significant inhibition of Akt and AMPK activities of the myocardium (Figure 4C, F and G). These results suggest that the myocardial glucose metabolism of mice is impaired after dCML. CS is the rate-limiting enzyme in the aerobic oxidation of glucose. The activity of CS was significantly inhibited in the dCML group (Figure 4H).

Exogenous CML inhibits glucose metabolism and promotes fibrosis, hypertrophy, and apoptosis in cardiomyocytes

Given that dCML inhibits myocardial glucose metabolism and promotes myocardial remodeling in mice, we further investigated the direct effects of CML *in vitro*. Exogenous stimulation with 10 mmol/L CML did not affect the viability of H9C2 cells (Figure 5A). Exogenous CML stimulation significantly decreased Glut-4 expression and significantly reduced the levels of Akt and AMPK phosphorylation in H9C2 cardiomyocytes, whereas the mRNA and protein levels of Glut-1 were significantly increased (Figure 5B-H). Immunocytochemical staining indicated that CML increased the content of collagen I in

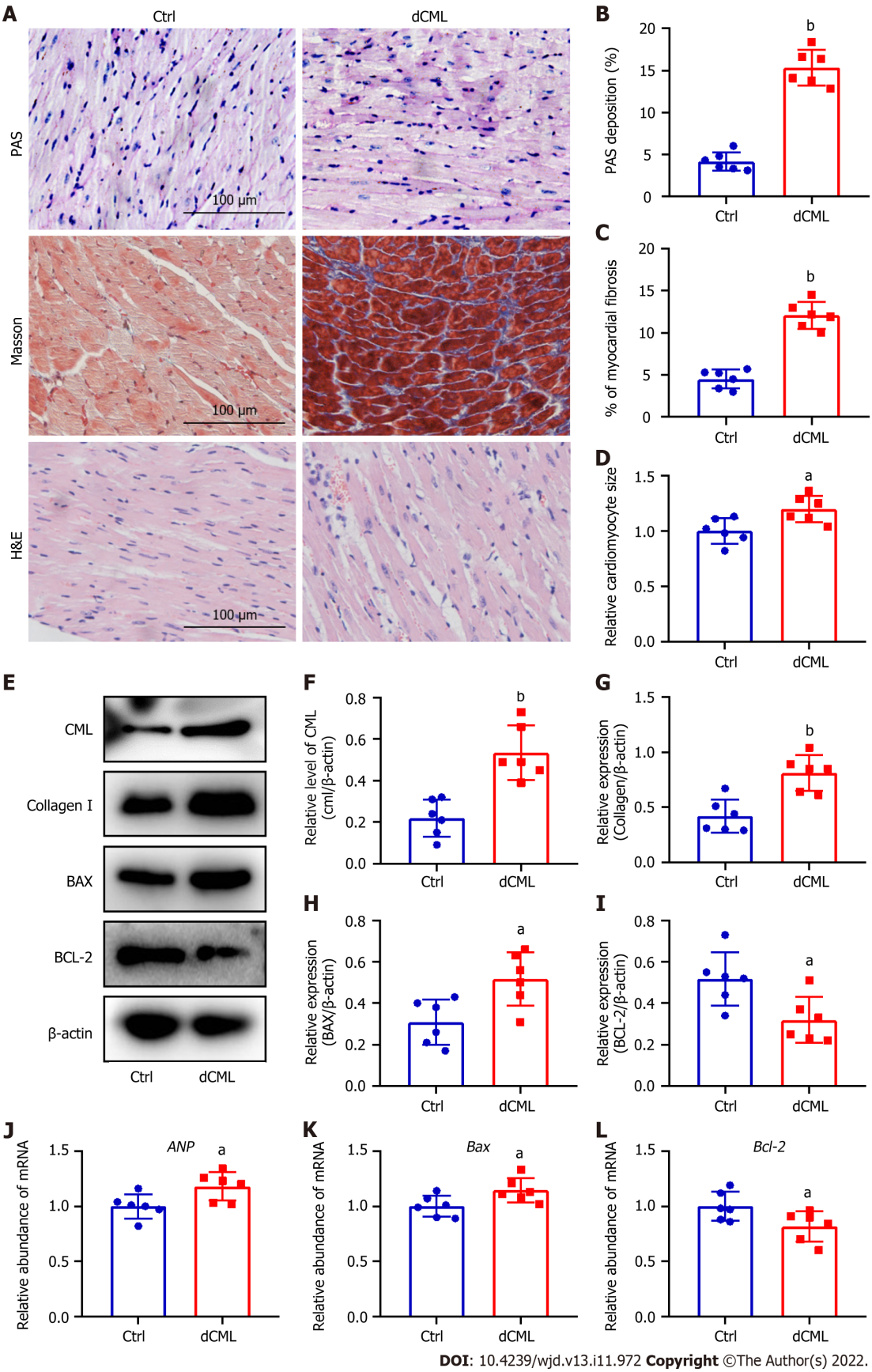

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Figure 3 Dietary Nᵋ-(carboxymethyl)lysine increases myocardial fibrosis, hypertrophy and apoptosis in mice. A: Mouse myocardial glycogen Periodic Acid Schiff (PAS) staining, Masson's trichrome staining, and hematoxylin and eosin staining; Scale 100 μm; B and C: Percentage of PAS-positive and fibrotic areas in the mouse myocardium; D: Relative area of myocardial cells in the myocardium; E-I: Western blotting and its relative level of Nᵋ-(carboxymethyl)lysine (CML), collagen I, B-cell leukemia/lymphoma 2 (Bcl-2) and Bcl-2-associated X (BAX) in the mouse myocardium; J-L: Atrial natriuretic peptide (*ANP*), *Bax*, and *Bcl-2* mRNA

levels in the mouse myocardium. dCML: Dietary CML. $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with the control (Ctrl) group.

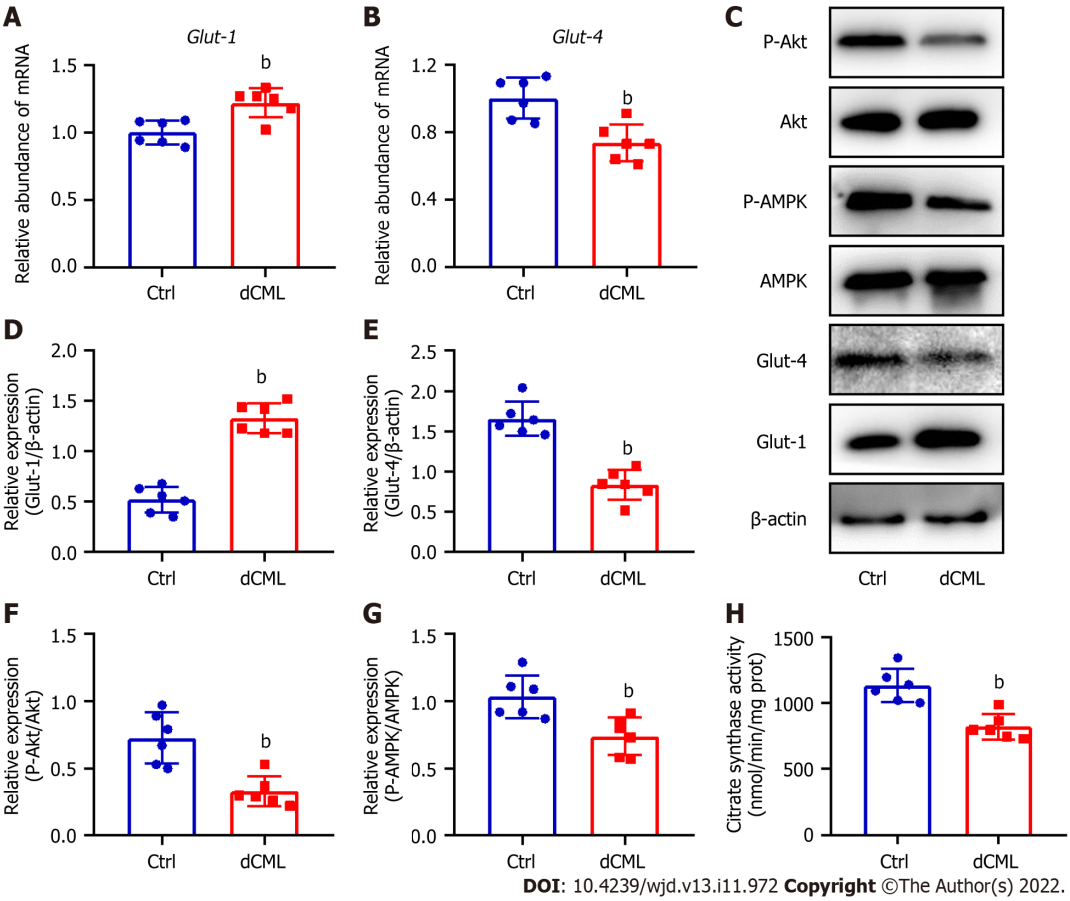

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Figure 4 Dietary N$^{\varepsilon}$-(carboxymethyl)lysine impairs glucose metabolism in mouse myocardium. A and B: mRNA levels of glucose transporter (*Glut*) *-1* and *Glut-4* in the mouse myocardium; C-G: Western blotting and its relative quantification of Glut-1, Glut-4, phospho-Akt, and phospho-AMP-activated protein kinase (AMPK) in the mouse myocardium; H: Citrate synthase (CS) activity in the mouse myocardium. dCML: Dietary N$^{\varepsilon}$-(carboxymethyl)lysine; Bcl-2: B-cell leukemia/lymphoma 2; BAX: Bcl-2-associated X; ANP: Atrial natriuretic peptide. $n = 6$. $^{b}P < 0.01$, compared with the control (Ctrl) group.

cardiomyocytes (Figure 5I and J). Phalloidin staining showed that the cardiomyocyte area in the CML group was significantly increased, and the expression of cardiac hypertrophy marker ANP was also significantly upregulated (Figure 5I, K and L). Meanwhile, the mRNA level of the apoptosis indicator *Bax* was increased in the CML group, and the level of the anti-apoptotic marker *Bcl-2* was significantly downregulated, suggesting that CML induced cardiomyocyte apoptosis (Figure 5M and N). The *Cs* mRNA level was also decreased in the CML group (Figure 5O).

DISCUSSION

This study investigated the effects of dCML on myocardial glucose metabolism and myocardial remodeling using experimental mouse models and cells. In the *in vivo* model, myocardial glucose uptake was tracked using ^{18}F-FDG micro-PET scans. The glucose uptake in the dCML group was significantly increased. Histological staining and detection of related molecular indicators suggested that dCML inhibited glucose metabolism in the myocardium and promoted myocardial fibrosis, cardiac hypertrophy, and apoptosis (Figure 6). *In vitro*, H9C2 cardiomyocytes were treated with exogenous CML to analyze changes in cardiac remodeling and glucose metabolism. Consistent with the *in vivo* evidence, CML inhibited glucose metabolism and promoted hypertrophy, collagen I expression, and apoptosis of cardiomyocytes. Our study may reveal new clues for underlying foodborne factors associated with myocardial injury and provide new ideas for the prevention and treatment of myocardial remodeling.

AGEs are a class of non-enzymatic reaction products composed of complex components, and the pathogenic role of AGEs has been previously reported[11,12]. The effects of AGEs may be receptor-

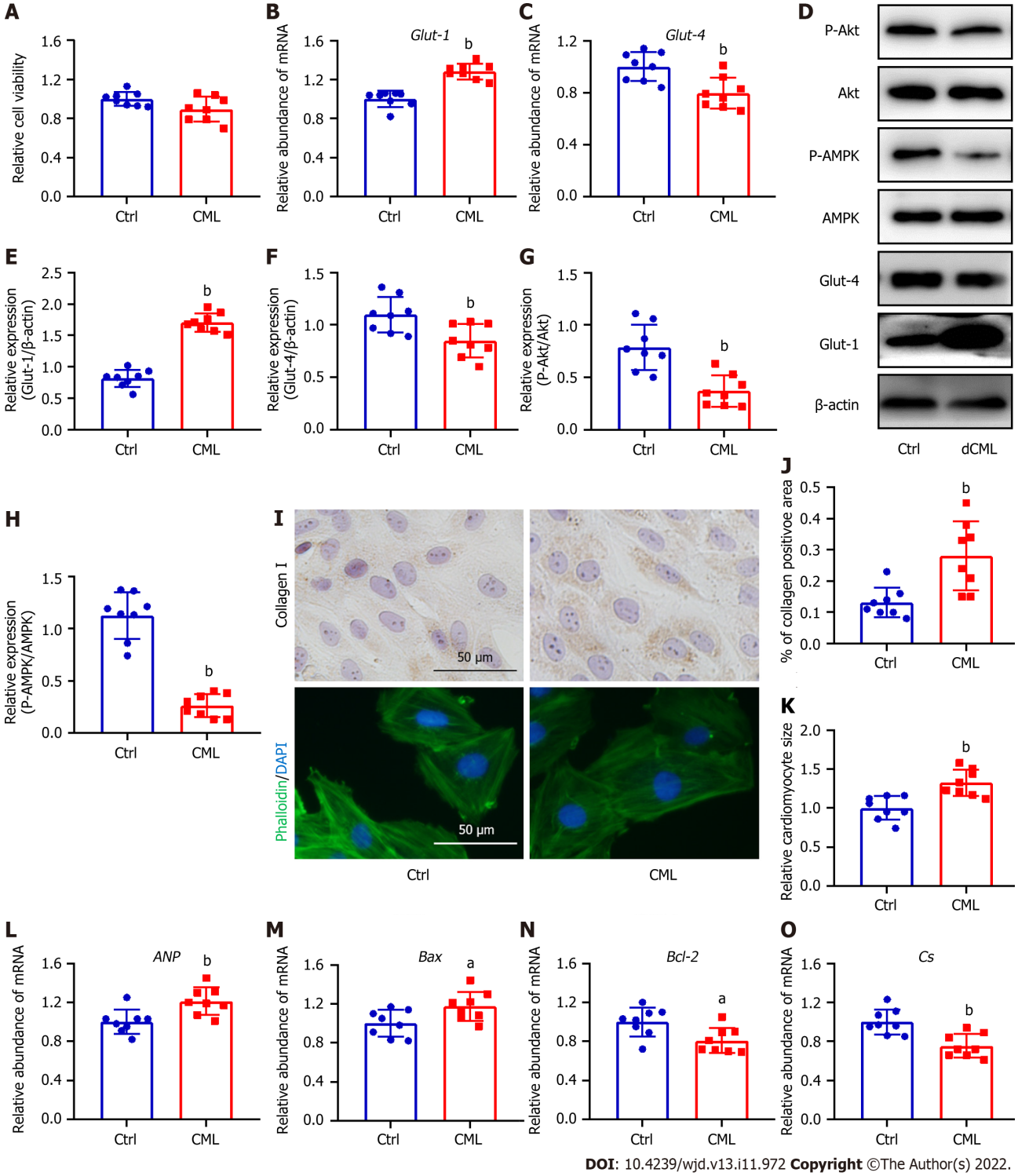

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Figure 5 Exogenous Nᵋ-(carboxymethyl)lysine inhibits the glucose metabolism and promotes collagen I expression, hypertrophy and apoptosis in H9C2 cells. A: Cell viability after the simulation of Nᵋ-(carboxymethyl)lysine (CML); B and C: Quantitative PCR detection of glucose transporter (*Glut*) *-1* and *Glut-4* mRNA; D-H: Relative expression of Glut-1, Glut-4, phospho-Akt, and phospho-AMP-activated protein kinase (AMPK) in H9C2 cells; I: Upper: Detection of collagen I content with immunocytochemical staining; bottom: Phalloidin-labeled H9C2 cardiomyocytes; J: Quantification of collagen I-positive areas; K: Quantitative analysis of cardiomyocyte area; L-O: Atrial natriuretic peptide *(ANP)*, Bcl-2-associated X *(Bax)*, B-cell leukemia/lymphoma 2 *(Bcl-2)*, and citrate synthase *(CS)* mRNA levels in H9C2 cardiomyocytes. dCML: Dietary CML. $n = 8$ independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with the control (Ctrl) group.

dependent or receptor-independent. In the receptor-independent pathway, AGEs cross-link with the extracellular matrix and change the physicochemical properties, which affects cell physiology and tissue function[36]. In the receptor-dependent pathway, AGEs bind to cell surface receptors, change the original signal transmission pathway, and lead to pathological outcomes[37]. In addition to AGEs, the precursors of AGEs, such as methylglyoxal, also accumulate in the body. These precursors can play a direct pathogenic role or continue to form AGEs[38]. Adverse effects of dietary AGEs have been

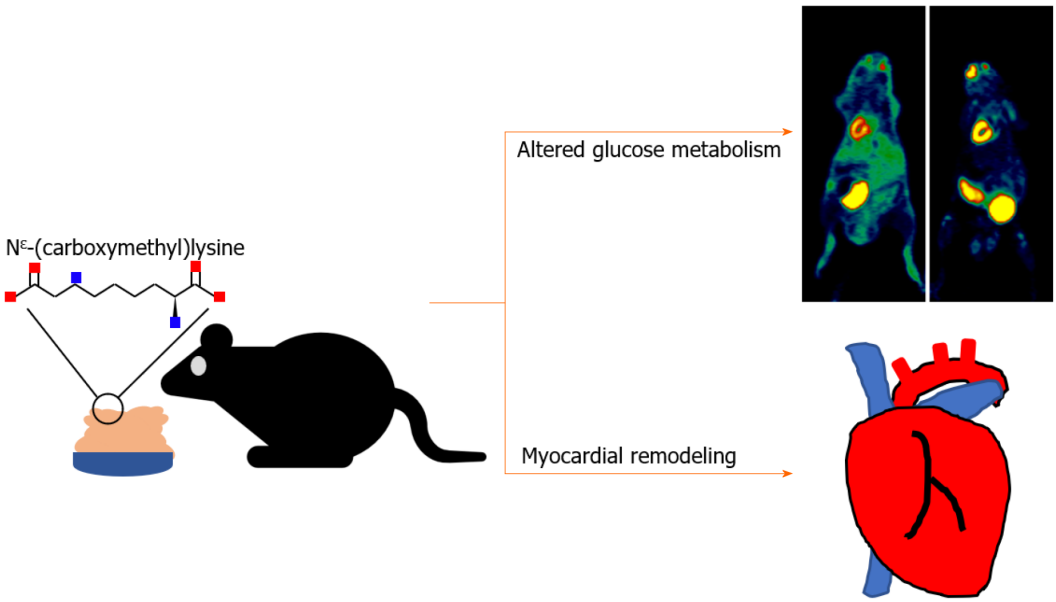

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Figure 6 Dietary Nᵋ-(carboxymethyl)lysine alters myocardial glucose metabolism and promotes myocardial remodeling.

previously reported. Wang *et al*[39] found that dietary AGEs disrupted gut microbiota and induced insulin resistance. Thornton *et al*[40] suggested that dietary AGEs affected ovarian function. A western diet rich in AGEs can also induce changes in the cardiovascular system[41]. Given that AGEs are multi-component, but whether each component has a similar effect is unclear. It is still unknown which component plays the most critical role. CML is one of the most active components of AGEs. Due to its ease of formation, CML is also found in high concentrations in food[42]. Therefore, our study explored the role of dCML, and showed that dCML can lead to disorders of myocardial glucose metabolism and myocardial remodeling. This finding may provide more evidence underlying the negative effects of foodborne AGEs.

Some studies have also reported the limited pathogenic role of dietary AGEs. Koyama *et al*[43] found no significant association between dietary AGEs and all-cause mortality in adults with diabetes. The double blind parallel study by Linkens *et al*[44] suggested that a short-term AGE diet did not affect the sensitivity, secretion and clearance of insulin, vascular function, and overall inflammation in individuals with abdominal obesity. The Maastricht Study also revealed that dietary AGEs are not associated with stiffness of the aorta or carotid arteries[45]. These studies are population-based, suggesting additional confounding factors than in animal models. Moreover, surveys of dietary structure or dietary interventions in these subjects were conducted for relatively short periods of time, which may not be sufficient to represent the long-term dietary habits of individuals. In our study, we focused on myocardial glucose uptake and its remodeling and found adverse effects of dCML. We also evaluated the systemic effects of dCML on mice. Exposure to 20 wk of dCML resulted in glucose intolerance and insulin resistance, but the fasting glucose did not reach the level of diabetes, suggesting a progressive pathogenic effect of dCML. It may take more than 20 wk of a CML diet to further increase blood glucose and worsen insulin resistance. Also, the weight gain of dCML mice became more obvious starting from the 12th wk. Prolonged dCML time may induce significant changes in mouse body weight.

Myocardium is one of the most energy-consuming organs, with 70% of the energy supply of adult myocardium derived from ATP produced by fatty acid oxidation, and glucose metabolism plays a secondary but important role[46]. Under physiological conditions, glucose is converted to pyruvate. ATP is produced *via* tricarboxylic acid cycle and respiratory chain. Under specific pathological conditions, glucose is the main energy substrate as a result of the reorganization of enzymes involved in energy metabolism. However, glycolysis is the main energy source rather than aerobic oxidation. The energy provided by glycolysis does not meet the long-term needs of myocardial activity. The overall cardiac metabolic activity is subsequently reduced, eventually leading to cardiomyocyte apoptosis and malignant remodeling of the myocardium[47]. However, an increase or decrease in myocardial glucose metabolism is also associated with specific pathological changes. For example, in diabetic cardiomyopathy, the lipotoxicity caused by diabetes increases the fatty acid metabolism in the heart, thus inhibiting glucose metabolism[48]. The interaction between fatty acids and glucose metabolism is also known as Randle Cycle[49]. However, the relationship between myocardial metabolic reprogramming and myocardial pathological remodeling is unclear. Whether myocardial metabolic disturbance is a cause or a consequence of myocardial remodeling is still inconclusive. In this study, we observed impaired myocardial glucose metabolism but increased glucose uptake after long-term dCML in mice.

The glucose metabolic pathways Akt and AMPK were significantly inhibited. The long-term dCML may alter the metabolic substrates for myocardial energy supply. CS, an enzyme initiating the tricarboxylic acid cycle, was also inhibited after exposure to dCML. Therefore, the myocardium has to absorb more glucose to provide adequate substrates for energy metabolism. The specific mechanism will be further explored in future studies.

To analyze the glucose uptake in the myocardium, we used micro-PET imaging based on the ^{18}F-FDG probe. Since ^{18}F-FDG was synthesized in 1969, it has been widely used in the diagnosis, staging and prognostic assessment of clinical diseases[50]. FDG is a glucose analog and is therefore involved in glucose processing *in vivo*. Under pathological conditions, inflammation or hypoxia can lead to impaired glucose metabolism but increased glucose uptake to provide adequate energy. Therefore, ^{18}F-FDG usually accumulates in the lesions. In the study of cardiovascular disease, ^{18}F-FDG imaging also plays an important role. ^{18}F-FDG is the reference standard for molecular imaging of myocardial inflammation[51]. Our study found an increased ^{18}F-FDG uptake but impaired glucose transport and metabolism in the myocardium of dCML mice, which may be related to the elevated levels of inflammation. Consistent with our study, in the spontaneously hypertensive rat model, myocardial ^{18}F-FDG imaging SUV was elevated, whereas glucose aerobic oxidation-related transporters and metabolic pathways were significantly inhibited[29].

We investigated the detrimental effects of dietary CML on myocardial remodeling to draw attention to the CML content in the diet. However, this study has some limitations. The exploration of specific mechanisms needs to be further continued in the future, and clinical evidence is also needed. We speculate that dietary CML may also promote myocardial remodeling through non-receptor and receptor approaches. CML could increase collagen cross-linking in the extracellular matrix and bind to its receptors to activate related signals. In the future, we will further explore the mechanism of cardiac remodeling induced by dietary CML to identify effective targets for intervention. There is an endogenous CML generation system in human body. Compared with reducing endogenous CML, reducing exogenous CML from dietary sources appears to be more controllable. Previous studies have also reported some CML inhibitors, such as antioxidants and aminoguanidine[15]. However, these additives may change the original methods of food production, and their high cost and unclear safety also limit the application. Therefore, in the future, we will also focus on strategies to inhibit CML in diet preparation.

CONCLUSION

Our study focused on the adverse effects of food-derived CML on myocardial glucose metabolism and remodeling. Long-term dCML leads to impaired myocardial glucose metabolism and induces myocardial hypertrophy, fibrosis, and apoptosis. This study offers new clues associated with myocardial remodeling and also provides an experimental basis for dietary planning to prevent cardiovascular disease prevention.

ARTICLE HIGHLIGHTS

Research background

N$^{\varepsilon}$-(carboxymethyl)lysine (CML), a major component of advanced glycation end products, exists in the daily diet and poses a threat to health after ingestion. It is necessary to evaluate the effect of dietary CML on the heart.

Research motivation

Previous studies have confirmed that the toxic metabolite CML can cause pathological changes in a variety of tissues such as blood vessels and bones. Foodborne CML, as the main source of CML, may lead to cardiac injuries.

Research objectives

To investigate the effects of dietary CML on cardiac remodeling and glucose metabolism.

Research methods

C57 BL/6 mice received a 20-wk CML diet (1 g/kg). The body weight, fasting blood glucose, fasting insulin and serum CML levels of mice were recorded. Exogenous CML was given to establish an *in vitro* H9C2 cell model. Micro-positron emission tomography was used to evaluate the glucose uptake of the mouse heart. Myocardial remodeling and glucose metabolism were detected by histological/cytological staining, Western blotting, and polymerase chain reaction.

Research results

The 20 wk of CML diet could cause insulin resistance in mice and increase CML levels in serum and heart. Myocardial fibrosis, hypertrophy and apoptosis in mice were significantly aggravated after dietary CML. Moreover, dietary CML increased myocardial glucose uptake but disrupted glucose metabolism. *In vitro,* exogenous CML inhibited glucose metabolism-related signaling pathways and promoted H9C2 cell hypertrophy, apoptosis and collagen I expression.

Research conclusions

Dietary CML promoted cardiac remodeling and abnormal glucose metabolism.

Research perspectives

This study emphasizes the cardiac hazards of dietary CML and provides new suggestions for the diet preparation in the prevention and treatment cardiovascular diseases.

ACKNOWLEDGEMENTS

We thank the Experimental Animal Center of Jiangsu University for guiding animal experiments.

FOOTNOTES

Author contributions: Sun Z designed the study and wrote the manuscript; Wang ZQ performed the experiments, analyzed the data, and prepared the images; all authors edited and approved the final version of the article.

Supported by the National Natural Science Foundation of China, No. 82070455; Natural Science Foundation of Jiangsu Province, No. BK20201225; Medical Innovation Team Project of Jiangsu Province, No. CXTDA2017010; and Research and Innovation Funding Project for College Students in Experimental Animal Center of Jiangsu University.

Institutional animal care and use committee statement: All animal experiments were approved by the Experimental Animal Use Ethics Committee of Jiangsu University.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: Filipodia
P-Editor: Chen YX

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 November 15; 13(11): 986-1000

DOI: 10.4239/wjd.v13.i11.986

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Observational Study

Risk factor analysis and clinical decision tree model construction for diabetic retinopathy in Western China

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Alsaidan A, Saudi Arabia; Laddha UD, India

Received: June 13, 2022
Peer-review started: June 13, 2022
First decision: August 1, 2022
Revised: August 20, 2022
Accepted: October 27, 2022
Article in press: October 27, 2022
Published online: November 15, 2022

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Abstract

BACKGROUND

Diabetic retinopathy (DR) is the driving force of blindness in patients with type 2 diabetes mellitus (T2DM). DR has a high prevalence and lacks effective therapeutic strategies, underscoring the need for early prevention and treatment. Yunnan province, located in the southwest plateau of China, has a high prevalence of DR and an underdeveloped economy.

AIM

To build a clinical prediction model that will enable early prevention and treatment of DR.

METHODS

In this cross-sectional study, 1654 Han population with T2DM were divided into groups without (n = 826) and with DR (n = 828) based on fundus photography. The DR group was further subdivided into non-proliferative DR (n = 403) and proliferative DR (n = 425) groups. A univariate analysis and logistic regression analysis were conducted and a clinical decision tree model was constructed.

RESULTS

Diabetes duration ≥ 10 years, female sex, standing- or supine systolic blood

pressure (SBP) ≥ 140 mmHg, and cholesterol ≥ 6.22 mmol/L were risk factors for DR in logistic regression analysis (odds ratio = 2.118, 1.520, 1.417, 1.881, and 1.591, respectively). A greater severity of chronic kidney disease (CKD) or hemoglobin A 1c increased the risk of DR in patients with T2DM. In the decision tree model, diabetes duration was the primary risk factor affecting the occurrence of DR in patients with T2DM, followed by CKD stage, supine SBP, standing SBP, and body mass index (BMI). DR classification outcomes were obtained by evaluating standing SBP or BMI according to the CKD stage for diabetes duration < 10 years and by evaluating CKD stage according to the supine SBP for diabetes duration ≥ 10 years.

CONCLUSION

Based on the simple and intuitive decision tree model constructed in this study, DR classification outcomes were easily obtained by evaluating diabetes duration, CKD stage, supine or standing SBP, and BMI.

Key Words: Diabetic retinopathy; Type 2 diabetes; Western China; Decision tree

Core Tip: Due to the underdeveloped economy and higher prevalence of diabetic retinopathy (DR), Yunnan province is facing a serious task of prevention. Based on a large sample of the Han population with type 2 diabetes mellitus in Yunnan province, this study constructed a cost-effective predictive model that may facilitate the timely and individualized estimation of DR risk.

Citation: Zhou YY, Zhou TC, Chen N, Zhou GZ, Zhou HJ, Li XD, Wang JR, Bai CF, Long R, Xiong YX, Yang Y. Risk factor analysis and clinical decision tree model construction for diabetic retinopathy in Western China. *World J Diabetes* 2022; 13(11): 986-1000

URL: https://www.wjgnet.com/1948-9358/full/v13/i11/986.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i11.986

INTRODUCTION

Type 2 diabetes mellitus (T2DM), a common chronic disease, poses a severe threat to human health and quality of life. By 2045, an estimated 552 million people worldwide will suffer from T2DM[1]. Given the socio-economic developments over the past 40 years, China has become increasingly urbanized, with a growing aging population. Moreover, obesity and overweight caused by lifestyle changes contribute to the growing burden of T2DM in China[2]. As a major vascular complication of T2DM, diabetic retinopathy (DR) is currently largely responsible for blindness in the working-class[3,4].

The two major challenges associated with DR include the high disease prevalence and lack of effective treatments. The global prevalence of DR is 34.6%. As of 2011, 126.6 million people suffered from DR, and this number is estimated to reach 191.0 million in 2030 without effective and timely measures[5]. The concerning prevalence and severity of DR worldwide may be modulated by racial/ethnic disparities, socio-economic status, health care systems, lifestyles, research methods, and other factors[6]. Regarding Asian populations, the prevalence of DR varies according to the region. For example, the prevalence of DR is 20.1%[7], 25.7%[8], and 35.0%[9] in Chinese Singaporeans, Chinese Americans, and Taiwanese Chinese, respectively. Further, the prevalence of DR in inland areas of China is 23%. It is also higher in rural areas than in urban areas and in northern areas than in southern and eastern coastal areas[10-12].

Nevertheless, there is currently a paucity of effective treatments for DR. In addition to systematic interventions for controlling blood glucose levels, blood pressure, and blood lipid levels, several modern therapies have been developed, such as laser photocoagulation[13] and intravitreal injections of anti-vascular endothelial growth factor (VEGF) antibodies or glucocorticoids, which can delay the progress of proliferative DR (PDR)[14,15]. However, several side effects associated with these therapies should be noted. For instance, photocoagulation may cause potential retinal damage, anti-VEGF injections are associated with relapse after drug withdrawal, and glucocorticoid use contributes to cataracts and elevated intraocular pressure in a considerable number of patients. Moreover, intraocular injections may cause complications such as endophthalmitis, intraocular hemorrhage, vitreous hemorrhage, and even retinal detachment[14,15]. Therefore, the utilization of these therapeutic options in clinical practice should be based on systematic evaluation and strict indications.

The high prevalence of DR and lack of efficient therapeutic strategies are associated with reduced quality of life of patients and pose a substantial socio-economic burden on individuals, families, and the society[16]. According to an analysis of the pedigree of T2DM in Yunnan province, the prevalence of DR [17] approximates the national average[10]. Therefore, the active search for associated risk factors is a fundamental priority for the prevention of DR. In this regard, a decision tree model established using identified risk factors is a useful tool. Distinct from traditional statistical methods such as logistic regression analysis, decision trees are effective machine-learning algorithms that solve classification problems. This method obtains a set of effective classification rules through systematic learning of multiple attributes of samples with known classification results. When faced with new unknown samples, the choice of classification or characteristic attributes can be quickly obtained based on the set of rules extracted from the established decision tree[18-20]. Thus, a decision tree is a prediction model with a simple and intuitive flowchart structure that is particularly suitable for use in clinical practice. This study examined the risk factors associated with DR in the Han population with T2DM in Yunnan province and constructed a clinical decision tree model.

MATERIALS AND METHODS

Study subjects

Patients from the Han population with T2DM were enrolled from the Department of Endocrinology, Affiliated Hospital of Yunnan University. All patients fulfilled the Chinese Diabetes Association criteria for the diagnosis of T2DM[21]. The criteria for exclusion were as follows: (1) Age < 18 years; (2) positive islet autoantibodies [including islet cell autoantibodies and autoantibodies to glutamic acid decarboxylase-65, insulin, the tyrosine phosphatases islet antigen 2 (IA-2) and IA-2β, and zinc transporter 8]; (3) acute complications of diabetes mellitus (including diabetic ketoacidosis or diabetic hyperosmolar state); (4) severe hepatic damage; (5) malignant tumors; (6) acute or chronic infectious diseases; (7) other eye diseases (including glaucoma, retinal vascular occlusion, and ischemic optic neuropathy); and (8) pregnancy. Finally, 1654 patients with T2DM were enrolled.

Ethical principles

This study was approved by the Ethics Committee of Affiliated Hospital of Yunnan University (No. 2021062), and written informed consent was obtained from all participants according to the principles of the Helsinki Declaration.

This trial registration was registered at ChiCTR (ChiCTR2100041888; registration on January 9, 2021, http://www.chictr.org.cn/index.aspx).

Clinical information collection

Patient sex, age, diabetes duration, height, weight, waist circumference, hip circumference, waist-hip ratio (WHR), body mass index (BMI), and systolic blood pressure (SBP) and diastolic blood pressure (DBP) (both in the standing and supine positions) were recorded.

Laboratory assessments

All patients fasted at 22:00 the day before blood collection. At 8:00 the next day, 6 mL of venous blood was collected. Fasting blood glucose (Glu0), hemoglobin A 1c (HbA1c), serum creatinine (Scr), blood urea nitrogen (BUN), uric acid (UA), triglycerides (Trig), cholesterol (Chol), high-density lipoprotein Chol (HDL-C), and low-density lipoprotein Chol (LDL-C) were measured. Based on the formula eGFR (mL/min/1.73 m^2) = 175 × Scr (mg/dL) - 1.234 × age - 0.179 × (0.790 for women)[22], the estimated glomerular filtration rate (eGFR) was calculated.

Ophthalmological measurements

All patients underwent non-mydriatic fundus photography and were evaluated according to the international clinical grading standards for DR established by the American Academy of Ophthalmology[23].

Definitions

According to the DR Preferred Practice Pattern[23]. DR was clinically classified into two types—non-PDR (NPDR) and PDR; the latter was identified by neovascularization and preretinal or vitreous hemorrhage. To further study the effect of blood pressure-related indicators (SBP and DBP in both the standing and supine positions), obesity-related indicators (BMI and waist circumference), blood glucose-related indicators (Glu0 and HbA1c), blood lipid-related indicators (Chol, Trig, LDL-C, and HDL-C), and renal function related indicators (UA and eGFR) on DR, these indicators were defined according to relevant guidelines or expert consensus. Abnormal blood pressure was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg[24]. Overweight was defined as BMI ≥ 24 kg/m^2, obesity was defined as BMI ≥ 28 kg/m^2, and abdominal obesity was defined as waist circumference ≥ 90 cm in men

or ≥ 85 cm in women[25]. Well-controlled blood glucose level was defined as Glu0 ≤ 7.0 mmol/L or HbA1c < 7%, generally controlled blood glucose level was defined as 7% ≤ HbA1c < 8%, and poorly controlled blood glucose level was defined as Glu0 > 7.0 mmol/L or HbA1c ≥ 8%[21]. Dyslipidemia was defined as Chol ≥ 6.22 mmol/L, Trig ≥ 2.26 mmol/L, or LDL-C ≥ 4.14 mmol/L and/or HDL-C < 1.04 mmol/L[26]. Hyperuricemia was defined as fasting serum UA > 420 μmol/L[27]. Stages of chronic kidney disease (CKD) were determined by eGFR as follows: CKD stage 1 (G1), eGFR ≥ 90 mL/min; CKD stage 2 (G2), eGFR = 60-89 mL/min; CKD stage 3 (G3), eGFR = 30-59 mL/min; CKD stage 4 (G4), eGFR = 15-29 mL/minl and CKD stage 5 (G5), eGFR < 15 mL/min [22].

Statistical analysis

Based on fundus photography, participants were divided into groups without DR (WDR group, n = 826) or with DR (DR group, n = 828). The DR group was further classified into the NPDR (n = 403) and PDR (n = 425) groups according to severity. All statistical analyses were performed using SPSS (SPSS 20.0, IBM, United States). Differences between the WDR and DR groups or the NPDR and PDR groups were assessed (Table 1). For continuous variables, Welch's t-test was used for normal distributions, while the Mann-Whitney U test was used for skewed distributions. For categorical variables, the chi-square test was performed. Using DR or PDR as the dependent variable, logistic regression analysis was explored to analyze DR or PDR-related risk factors. Logistic regression analysis was performed using forward selection (likelihood ratio), with P < 0.05 as the entry criterion and P > 0.1 as the removal criterion. The decision tree method was performed using the chi-squared automatic interaction detector, with 70% participants as the training dataset, and the remaining 30% as the test dataset. The variable assignments used in both logistic regression analysis and decision tree model are presented in Table 2.

RESULTS

Baseline clinical characteristics

The baseline clinical characteristics of all participants are presented in Table 1.

Univariate analysis of DR-related risk factors

As shown in Table 1, the proportion of women, diabetes duration, supine SBP, standing SBP, supine DBP, Chol, BUN, UA, Scr, Glu0, and HbA1c were higher in the DR group than in the WDR group (all P values < 0.05). In contrast, the DR group had a lower BMI, hip circumference, and eGFR than the WDR group (all P values < 0.05).

Univariate analysis of PDR-related risk factors

The proportion of women, supine SBP, supine DBP, Chol, LDL-C, and BUN were significantly higher in the PDR group than in the NPDR group (all P values < 0.05). However, age, hip circumference, and eGFR were significantly lower in the PDR group than in the NPDR group (all P values < 0.05) (Table 1).

Logistic regression analysis of DR-related risk factors

Women had a 1.520 times higher risk of DR [95% confidence interval (CI): 1.218 to 1.897] compared to men (Figure 1). Patients with diabetes duration ≥ 10 years had a 2.118-fold higher risk of DR than those with diabetes duration < 10 years (95%CI: 1.661 to 2.700). The risk of DR in patients with standing SBP ≥ 140 mmHg was 1.417 times higher than that in patients with standing SBP < 140 mmHg (95%CI: 1.046 to 1.919). Compared to patients with supine SBP < 140 mmHg, those with supine SBP ≥ 140 mmHg had a 1.881-fold higher risk of DR (95%CI: 1.399 to 2.528). Compared to patients with normal Chol, those with Chol ≥ 6.22 mmol/L had a 1.591 times higher risk of DR (95%CI: 1.104 to 2.291). The risk of DR in patients with CKD stages G2, G3, G4, and G5 was 2.206 (95%CI: 1.678 to 2.899), 7.860 (95%CI: 4.573 to 13.512), 9.693 (95%CI: 3.255 to 28.862), and 20.691 (95%CI: 2.540 to 168.581) times higher than that in patients with CKD stage G1. In other words, a greater severity of CKD was associated with a higher risk of DR. The risk of DR in patients with 7% ≤ HbA1c < 8% or HbA1c ≥ 8% was 1.787 (95%CI: 1.198 to 2.664) and 3.073 (95%CI: 2.225 to 4.245) times higher than that in patients with HbA1c < 7%, indicating that worse control of HbA1c was associated with a higher risk of DR.

Logistic regression analysis of PDR-related risk factors

Compared to men, women had a 2.161-fold higher risk of progression to PDR (95%CI: 1.615 to 2.890) (Figure 2). Compared to patients with diabetes duration < 10 years, patients with diabetes duration ≥ 10 years had a 1.483 times higher risk of PDR (95%CI: 1.099 to 2.001). The risk of progression to PDR in patients with CKD stages G3 and G4 was 2.109 (95%CI: 1.362 to 3.266) and 2.290 (95%CI: 1.016 to 5.165) times higher than that in patients with CKD stage G1.

Decision tree modeling of DR-related risk factors

As shown in Figure 3, the importance of variables in the decision tree model was presented as a root-to-

Table 1 Univariate analysis of risk factors for diabetic retinopathy or proliferative diabetic retinopathy

Variable	WDR group (*n* = 826)	DR group (*n* = 828)	NPDR group (*n* = 403)	PDR group (*n* = 425)	*P* value[1]	*P* value[2]
Age (yr)	53.9 ± 10.3	54.9 ± 10.7	56.2 ± 10.9	53.6 ± 10.3	0.056	< 0.001
Sex (male/female)	487/339	424/404	238/165	186/239	0.002	< 0.001
Diabetes duration (yr)	6.9 ± 5.2	11.2 ± 6.8	10.9 ± 7.1	11.4 ± 6.4	< 0.001	0.221
Height (cm)	165.2 ± 8.3	162.5 ± 8.3	164.2 ± 8.5	160.8 ± 7.8	< 0.001	< 0.001
Weight (kg)	68.1 ± 11.7	64.2 ± 11.3	65.7 ± 11.5	62.8 ± 10.9	< 0.001	< 0.001
BMI (kg/m^2)	24.9 ± 3.3	24.3 ± 3.4	24.3 ± 3.3	24.2 ± 3.5	< 0.001	0.880
Waist circumference (cm)	90.8 ± 9.5	89.9 ± 10.0	90.2 ± 9.7	89.6 ± 10.3	0.060	0.399
Hip circumference (cm)	97.6 ± 7.6	95.9 ± 7.4	96.4 ± 7.2	95.4 ± 7.6	< 0.001	0.046
WHR	1.0 ± 0.5	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.284	0.092
Standing SBP (mmHg)	129.0 ± 16.9	136.4 ± 22.9	135.9 ± 22.6	136.9 ± 23.2	< 0.001	0.517
Standing DBP (mmHg)	83.4 ± 11.6	83.8 ± 13.4	83.5 ± 13.0	84.0 ± 13.7	0.501	0.578
Supine SBP (mmHg)	130.1 ± 16.3	141.0 ± 21.3	139.4 ± 20.6	142.4 ± 22.0	< 0.001	0.045
Supine DBP (mmHg)	82.6 ± 11.2	85.0 ± 12.3	83.9±12.0	86.1 ± 12.4	< 0.001	0.012
Glu0 (mmol/L)	9.4 ± 3.3	9.9 ± 3.7	10.0 ± 3.7	9.8 ± 3.7	0.008	0.647
HbA1c (%)	9.1 ± 2.5	9.8 ± 2.4	9.9 ± 2.5	9.7 ± 2.3	< 0.001	0.402
Chol (mmol/L)	4.4 ± 1.5	5.0 ± 1.5	4.8 ± 1.5	5.1 ± 1.4	< 0.001	0.013
Trig (mmol/L)	2.8 ± 2.3	2.6 ± 2.4	2.6 ± 2.5	2.6 ± 2.4	0.033	0.856
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	0.057	0.095
LDL-C (mmol/L)	2.9 ± 0.9	3.0 ± 1.0	2.9 ± 1.0	3.1 ± 1.1	0.101	0.004
BUN (μmol/L)	5.1 ± 1.7	6.6 ± 2.8	6.4 ± 2.8	6.8 ± 2.8	< 0.001	0.021
UA (μmol/L)	347.6 ± 103.4	359.4 ± 102.0	355.4 ± 106.4	363.2 ± 97.6	0.019	0.270
Scr (μmol/L)	69.8 ± 28.3	90.0 ± 61.8	86.0 ± 64.1	93.9 ± 59.4	< 0.001	0.068
eGFR (mL/min)	116.9 ± 37.3	98.3 ± 42.4	103.5 ± 40.9	93.3 ± 43.2	< 0.001	0.001

[1]WDR group *vs* DR group.
[2]NPDR group *vs* PDR group.
WDR: Without diabetic retinopathy; DR: Diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; BMI: Body mass index; WHR: Waist-hip ratio; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Glu0: Fasting blood glucose; HbA1c: Glycated hemoglobin A1c; Chol: Cholesterol; Trig: Triglyceride; HDL-C: High-density lipoprotein Chol; LDL-C: Low-density lipoprotein Chol; BUN: Blood urea nitrogen; UA: Uric acid; Scr: Serum creatinine; eGFR: Estimated glomerular filtration rate.

leaf structure, with diabetes duration being the first variable or root node, followed by CKD stage, supine SBP, standing SBP, and BMI, in order of importance. As presented in Table 3, seven "if-then" rules summarized the path from the root node to each leaf node.

DISCUSSION

In this study, we attempted to reveal the DR-related risk factors in Han population with T2DM in Yunnan province and construct a predictive model for personalized DR risk assessment and early preventive effect.

Studies have reported that various factors modulate the effects of age on DR. Although sporadic cases have been reported, the onset of DR before puberty is extremely rare[28,29]. Researchers have suggested that patients with diabetes during adolescence are prone to develop serious vascular complications, including DR, compared to patients with diabetes after adolescence. This could be partly due to the characteristics of adolescent patients; for example, patients at this stage tend to be accompanied by dramatic hormone level fluctuations, and most patients present with type 1 diabetes tend to have relatively poor blood glucose self-management ability[30]. Therefore, this study focused on Han

Table 2 Variable assignment

Variable	Assignment
Sex	0 = male
	1 = female
Age	0 = age < 60 years
	1 = age ≥ 60 years
Diabetes duration	0 = duration < 10 years
	1 = duration ≥ 10 years
Blood pressure	0 = normal (SBP < 140 mmHg and/or DBP < 90 mmHg)
	1 = abnormal (SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg)
BMI	0 = normal (BMI < 24 kg/m^2)
	1 = overweight (24 kg/m^2 ≤ BMI < 28 kg/m^2)
	2 = obesity (BMI ≥ 28 kg/m^2)
Waist circumference	0 = normal (male < 90 cm, female < 85 cm)
	1 = increased (male ≥ 90 cm, female ≥ 85 cm)
Glu0	0 = well controlled (Glu0 ≤ 7.0 mmol/L)
	1 = poorly controlled (Glu0 > 7.0 mmol/L)
HbA1c	0 = well controlled (HbA1c < 7%)
	1 = generally controlled (7% ≤ HbA1c < 8%)
	2 = poorly controlled (HbA1c ≥ 8%)
Blood lipids	0 = normal
	1 = dyslipidemia (met any of the following criteria: TG ≥ 2.26 mmol/L; TC ≥ 6.26 mmol/L; LDL ≥ 4.14 mmol/L; HDL < 1.04 mmol/L)
UA	0 = normal
	1 = hyperuricemia (UA > 420 μmmol/L)
CKD stage	0 = G1 (eGFR ≥ 90 mL/min)
	1 = G2 (eGFR = 60-89 mL/min)
	2 = G3 (eGFR = 30-59 mL/min)
	3 = G4 (eGFR = 15-29 mL/min)
	4 = G5 (eGFR < 15 mL/min)

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; Glu0: Fasting blood glucose; HbA1c: Glycated hemoglobin A1c; Trig: Triglyceride; Chol: Cholesterol; LDL-C: Low-density lipoprotein Chol; HDL-C: High-density lipoprotein Chol; UA: uric acid; CKD: Chronic kidney disease; G: Grade; eGFR: Estimated glomerular filtration rate.

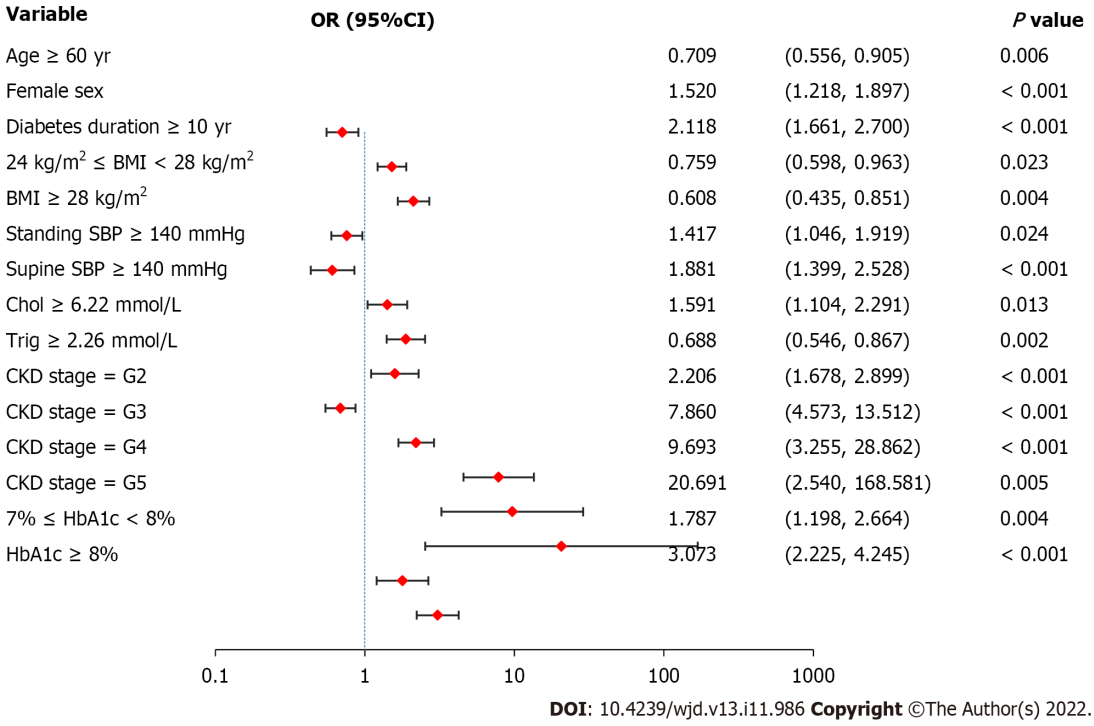
population with T2DM aged ≥ 18 years in Yunnan province to exclude the potential confounding effects of adolescence and type 1 diabetes on the results.

DR has emerged as the leading cause of blindness among 27-75 year olds worldwide[3,21]. A Chinese meta-analysis reported that the prevalence of DR in patients with T2DM was age related, increasing from 12.55% in adults aged 30-39 years to 20.44% in adults aged 60-69 years and decreasing to 11.22% in those aged ≥ 80 years[12]. Of the 1654 patients enrolled in this study, 33.49% and 16.56% patients with DR were < 60 years of age (n = 554) and ≥ 60 years of age (n = 274), respectively. This suggests that the peak of DR prevalence in Han population with T2DM in Yunnan province is concentrated in the population aged < 60 years, which accounts for the majority of the social labor force. In addition, univariate analysis (Table 1) revealed no significant difference in age between the DR and WDR groups (P = 0.056). However, overall age was dramatically lower in the PDR group than in the NPDR group (P < 0.001). As shown in Figures 1 and 2, age was not retained in the logistic regression equation. In conclusion, the correlation between age and DR is complex; this association depends on age stratification and may be affected by the degree of vision. This relationship warrants further exploration in future studies.

Table 3 "If-then" rules extracted from decision tree

Rule	If	Then
R1	Diabetes duration < 10 years, CKD stage = G1, standing SBP < 140 mmHg	Then: WDR
R2	Diabetes duration < 10 years, CKD stage = G1, standing SBP ≥ 140 mmHg	Then: DR
R3	Diabetes duration < 10 years, CKD stage = G2, BMI < 24 kg/m^2	Then: DR
R4	Diabetes duration < 10 years, CKD stage = G2, BMI ≥ 24 kg/m^2	Then: WDR
R5	Diabetes duration < 10 years, CKD stage = G3/G4/G5	Then: DR
R6	Diabetes duration ≥ 10 years, supine SBP < 140 mmHg, CKD staging = G1/G5	Then: WDR
R7	Diabetes duration ≥ 10 years, supine SBP < 140 mmHg, CKD staging = G2/G3/G4	Then: DR
R8	Diabetes duration ≥ 10 years, supine SBP ≥ 140 mmHg, CKD staging = G1/G2	Then: WDR
R9	Diabetes duration ≥ 10 years, supine SBP ≥ 140 mmHg, CKD staging = G3/G4/G5	Then: DR

R: Rule; CKD: Chronic kidney disease; SBP: Systolic blood pressure; WDR: Without diabetic retinopathy; DR: Diabetic retinopathy.



DOI: 10.4239/wjd.v13.i11.986

Figure 1 Logistic regression analysis of diabetic retinopathy-related risk factors. Female sex, diabetes duration ≥ 10 years, standing systolic blood pressure (SBP) ≥ 140 mmHg, supine SBP ≥ 140 mmHg, cholesterol ≥ 6.22 mmol/L, greater severity of chronic kidney disease, and worse control of hemoglobin A 1c are associated with a higher risk of diabetic retinopathy. Values are shown using a base 10, logarithmic scale. DR: Diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; SBP: Systolic blood pressure; Chol: Cholesterol; Trig: Triglyceride; CKD: Chronic kidney disease; HbA1c: Glycated hemoglobin A1c.

The relationship between sex and DR is unclear. A study from Germany and Australia based on 120000 samples suggested that women are more likely to develop DR than men[31]. Similarly, studies in the United Kingdom and Japan have reported that women are more prone to visual impairments than men[32,33]. Other studies from the United States and India, however, have reported that the men have a higher risk of DR than women[6,34,35]. In particular, the United Kingdom Prospective Diabetes Study (UKPDS) has proposed that DR progression is associated with the male sex[36]. Therefore, it is necessary to further explore the correlation between sex and DR. Univariate analysis (Table 1) revealed that the proportion of women was significantly higher in the DR group than in the WDR group (P = 0.002). Moreover, the proportion of women was significantly higher in the PDR group than in the NPDR group (P < 0.001). Indeed, further logistic regression analysis (Figures 1 and 2) re-emphasizes the importance of female sex. These findings suggest that female sex not only is a risk factor for the development of DR in patients with T2DM but also contributes to the progression of DR to PDR, at least

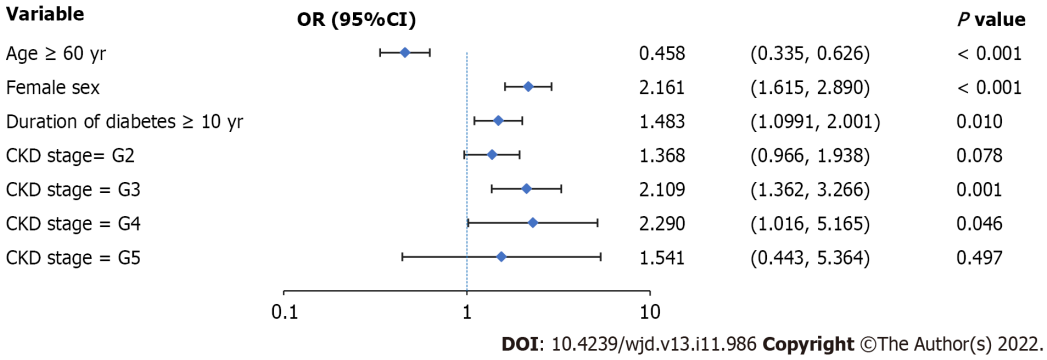


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Figure 2 Logistic regression analysis of proliferative diabetic retinopathy-related risk factors. Female sex, diabetes duration ≥ 10 years, and chronic kidney disease stage G3 or G4 are risk factors for the progression to proliferative diabetic retinopathy. Values are shown using a base 10, logarithmic scale. Abbreviations: PDR: Proliferative diabetic retinopathy; NPDR: Non-proliferative diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; CKD: Chronic kidney disease.

in Yunnan province.

It is well-established that the diabetes duration majorly affects the occurrence and progression of DR [23,37]. A case-control study in South Korea demonstrated that of 523 patients with T2DM, 44.9% developed DR, and 13.6% developed PDR. The average diabetes duration to mild NPDR, moderate-severe NPDR, and PDR was 14.8, 16.7, and 17.3 years, respectively[38]. Based on the Chinese population, a meta-analysis indicated that the prevalence of DR in patients newly diagnosed with diabetes and patients with a disease course of ≥ 10 years was 9.00% and 55.52%, respectively[12]. Univariate analysis (Table 1) revealed that the diabetes duration in the DR group was substantially longer than that in the WDR group ($P < 0.001$). In contrast, the diabetes duration did not differ significantly between the PDR and NPDR groups ($P = 0.221$). However, logistic regression analysis (Figures 1 and 2) revealed that a diabetes duration ≥ 10 years was an extremely risk for the occurrence and progression of DR. Crucially, the diabetes duration was classified as the root node of the DR decision tree model (Table 3 and Figure 3), emphasizing that the diabetes duration is critical in DR risk assessment.

In addition to the diabetes duration, good glycemic control is considered a key factor for reducing vascular complications of diabetes[39]. This study focused on two blood-glucose-related indicators, Glu0 and HbA1c. Compared with the transient characteristics of Glu0, HbA1c reflects the overall level of blood glucose control of patients in the prior 2 to 3 months. In this study, univariate analysis (Table 1) indicated that both Glu0 and HbA1c were substantially greater in the DR group than in the WDR group (all $P < 0.05$). As shown in Figure 1, HbA1c but not Glu0 was retained in the logistic regression equation. These findings suggest that poor HbA1c control is associated with a higher risk of DR. In conclusion, compared to Glu0, HbA1c, which reflects long-term glucose control levels, is more relevant for the prevention of DR. However, HbA1c did not negatively affect the progression of DR. Of note, large clinical studies such as the UKPDS[40,41] and the Diabetes Control and Complications Study[42] have demonstrated that early and intensive glucose control can reduce the occurrence and progression of diabetic vascular complications, including DR. However, good glycemic control is not equivalent to excessive control. Indeed, extensive evidence indicates that recurrent hypoglycemic episodes caused by excessive strict glycemic control with insulin are associated with the early deterioration of DR, but the underlying mechanisms are unclear[43-45].

Previous studies have demonstrated that hypertension is linked to the development and severity of DR[46,47]. Since patients with diabetes are prone to have complications of postural blood pressure changes[48,49], data on standing and supine blood pressure were collected simultaneously. Univariate analysis (Table 1) revealed that standing or supine SBP and supine DBP were significantly lower in the WDR group than in the DR group (all $P < 0.001$), but there was no obvious difference in standing DBP between the groups ($P = 0.501$). Although supine SBP and supine DBP were lower in the NPDR group than in the PDR group (all $P < 0.05$), no significant intergroup differences existed in standing SBP and standing DBP (all $P > 0.05$). Further logistic regression analysis (Figures 1 and 2) indicated that SBP had an effect on DR occurrence but not DR progression. In addition, supine SBP ≥ 140 mmHg was the leaf node of the decision tree model, second only to diabetes duration. Our results emphasize the detrimental effects of elevated SBP (especially supine SBP) on DR, suggesting that good blood pressure control is vital for the prevention of DR. Furthermore, the benefits of certain antihypertensive drugs, particularly angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, are not limited to lowering blood pressure[50,51] and may also benefit DR through neuroprotection[52,53], increasing insulin sensitivity[54], anti-inflammatory effects[55], and inhibiting the blood-eye barrier[56,57].

To date, the complex link between dyslipidemia and DR has remained controversial[10,47,58,59]. In this study, the occurrence and development of DR seemed to be more strongly affected by Chol than by

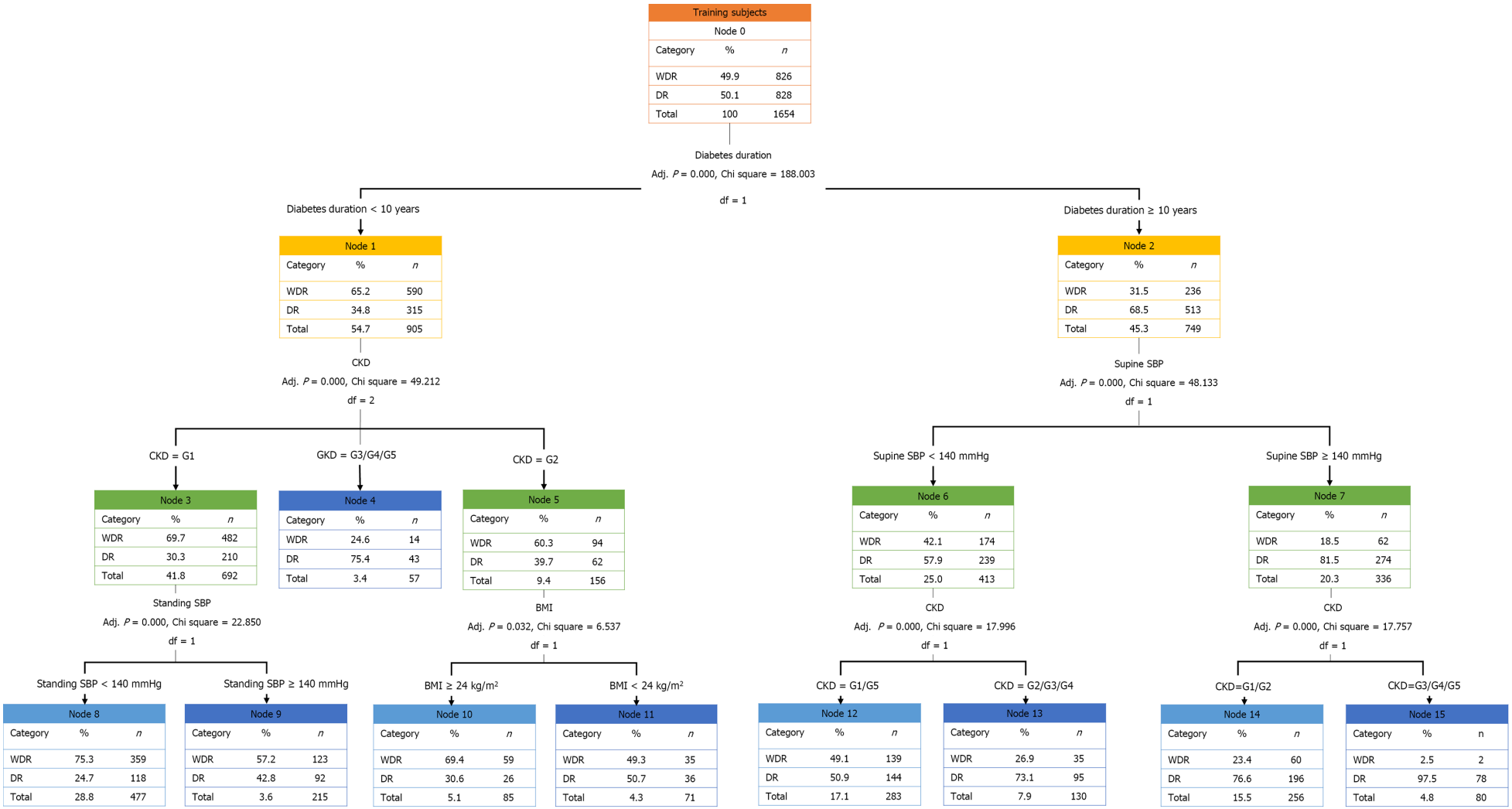

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Figure 3 Training dataset of decision tree model for diabetic retinopathy. Based on the decision tree model constructed in this study, the diabetic retinopathy classification outcomes are obtained by evaluating standing systolic blood pressure (SBP) or body mass index according to the chronic kidney disease (CKD) stage for patients with a diabetes duration < 10 years and the evaluation of CKD stage according to the supine SBP for patients with a diabetes duration ≥ 10 years. WDR: Without diabetic retinopathy; DR: Diabetic retinopathy; CKD: Chronic kidney disease; SBP: Systolic blood pressure; BMI: Body mass index.

Trig, HDL-C, and LDL-C Univariate analysis (Table 1) revealed that Chol was significantly higher in the DR group than in the WDR group ($P < 0.001$) and significantly higher in the PDR group than in the NPDR group ($P = 0.013$). Moreover, as shown in Figures 1 and 2, Chol increased the risk of developing DR in patients with T2DM, but had no remarkable impact on DR progression. A recent meta-analysis revealed that lipid-lowering drugs exerted a protective effect on the progression of DR[60] but did not affect the deterioration of visual acuity or aggravation of hard exudate. Therefore, further large-scale clinical trials are urgently needed to substantiate the necessity of early application of lipid-lowering drugs in patients with DR.

In parallel with the tremendous rise in the global prevalence of obesity, the prevalence of obesity-related T2DM has also increased annually[2]. However, the relationship between obesity and DR has not been fully elucidated. Reports from Wisconsin illustrated that obesity (defined by BMI) was not independently implicated in the occurrence or progression of DR in patients with T2DM within 10 years [61]. Similarly, in the Hoorn study, WHR, but not BMI, was related to the occurrence of DR[62]. However, data based on Asian populations have provided the opposite conclusions. For example, based on a sample of 420 Asian patients with T2DM, Man *et al*[63] reported that BMI was negatively correlated with mild, moderate, and severe DR, but WHR was positively correlated with DR severity. Subsequently, a Korean study demonstrated that higher BMI, increased waist circumference, and higher body fat content (measured by dual-energy X-ray) were notably correlated with a lower risk of DR[64]. In this study, data regarding relevant body mass indicators were collected, including those on BMI, waist circumference, hip circumference, and WHR. Univariate analysis (Table 1) revealed that BMI and hip circumference in the DR group were remarkably lower than those in the WDR group (all $P < 0.001$), but waist circumference was did not differ across the groups ($P = 0.060$). Although the PDR group had a lower hip circumference than the NPDR group ($P = 0.046$), there were no significant intergroup differences in BMI and waist circumference ($P > 0.05$). In addition, an obvious difference in WHR was not noted among groups (all $P > 0.05$). Although the logistic regression model ultimately did not retain BMI, waist circumference, hip circumference, and WHR (Figures 1 and 2), the decision tree model (Table 3 and Figure 3) supported the protective effect of higher BMI for the assessment of DR risk. In general, although the relationship between these obesity-relevant indicators and DR is yet to be confirmed, it can be conjectured that excessively low BMI is not conducive to protection against DR in the Asian population.

Among BUN, Scr, and UA, eGFR calculated using Scr is the gold standard for CKD staging[65]. In addition, CKD staging is an essential approach to evaluating the severity of diabetic nephropathy in clinical practice. Univariate analysis (Table 1) indicated that eGFR was significantly lower in the DR group than in the WDR group ($P < 0.001$) and was significantly lower in the PDR group than in the NPDR group ($P = 0.001$). Furthermore, Figures 1 and 2 highlight the importance of CKD staging in the risk assessment of DR. In this regard, the risk of occurrence and progression of DR increased stepwise with each additional risk level of CKD staging. Notably, in the decision tree model (Table 3 and Figure 3), CKD staging was second only to diabetes duration for DR risk assessment. In particular, CKD staging is a key indicator of diabetic nephropathy, and the current results also suggest that DR is often comorbid with diabetic nephropathy. This highlights the need to simultaneously screen for diabetic nephropathy in patients with DR.

Differing from traditional statistical methods such as logistic regression analysis, decision trees are successfully employed in the field of medicine with its advantages in solving classification problems, that is, qualitatively judging the possibility of each risk factor at a specific level. Decision trees obtain a set of effective classification rules by systematically learning multiple attributes of the samples with known classification results. When faced with new unknown samples, the appropriate classification or characteristic attributes can be quickly obtained based on the set of rules extracted from the established decision tree[18-20]. In other words, three basic elements compose the decision tree: root node, internal node, and leaf node. The root node is the main feature attribute in the model, the internal node is the secondary attribute judgment based on the root node, and the leaf node is the final classification outcome of the model.

This study extended traditional statistical analysis by building a DR decision model using machine learning based on the attributes of T2DM samples. In the decision tree model, diabetes duration was demonstrated to primarily affect the occurrence of DR in patients with T2DM, namely, the root node. The extraction rules were interpreted as follows: for patients with diabetes duration < 10 years, if they met the criteria of: (1) CKD stage = G3/G4/G5; (2) CKD stage = G2 and BMI < 24 kg/m^2; or (3) CKD stage = G1 and standing SBP ≥ 140 mmHg, then DR was prone to occur. In contrast, for patients with diabetes duration ≥ 10 years, if they met the criteria of: (1) Supine SBP < 140 mmHg and CKD stage = G2/G3/G4; or (2) supine SBP ≥ 140 mmHg and CKD stage = G3/G4/G5, then DR was prone to occur. This model may assist clinicians in Yunnan province (particularly primary medical staff who lack relevant DR detection approaches such as ophthalmoscope) to make more effective clinical predictions of DR risk in patients with T2DM. Our decision tree model is simple and intuitive, highlighting its potential for application in clinical practice.

However, there are several limitations that should be noted. First of all, this study did not record in detail the medication of patients, especially the use of anti-diabetic, lipid-lowering and antihypertensive drugs. Secondly, Yunnan province is located in the western plateau of China, and its climate, cultural

and economic conditions are very different from those of plain areas. Therefore, these confounding factors should be included in the future to enhance the integrity and reliability of research conclusions.

CONCLUSION

Female sex, diabetes duration ≥ 10 years, standing or supine SBP ≥ 140 mmHg, Chol ≥ 6.22 mmol/L, deterioration of CKD stage, and HbA1c are key DR-related risk factors in the Han population with T2DM in Yunnan province. The concise and intuitive DR prediction model developed through machine learning in this study could help clinicians quickly predict DR outcomes based on patients' potential risk factors and conduct early individualized interventions.

ARTICLE HIGHLIGHTS

Research background

Yunnan province has a high prevalence of diabetic retinopathy (DR). Accordingly, it is of great significance to explore the DR-related factors and to construct an economic and intuitive clinical prediction model.

Research motivation

The research motivation is early intervention using the DR-related risk factors from the perspective of a predictive model to reduce the prevalence of DR in patients with type 2 diabetes mellitus (T2DM).

Research objectives

The research intends to establish a prediction model that allows clinically early prevention and treatment of DR.

Research methods

A total of 1654 Han population with T2DM were recruited in this study and were grouped in the without DR and DR groups. The DR group was further subgrouped according to the severity of DR. Then, univariate analysis, logistic regression analysis, and clinical decision tree models of clinical data were performed.

Research results

Based on the decision tree model constructed in this study, DR classification outcomes were obtained by evaluating diabetes duration followed by stages of chronic kidney disease, supine systolic blood pressure (SBP), standing SBP, and body mass index.

Research conclusions

Personalized interventions for DR-related risk factors based on a decision tree model may potentially reduce the prevalence of DR.

Research perspectives

In this study, patients with T2DM in Western China were taken as samples to analyze the influencing factors of DR and build a clinical prediction model. In the future, it is hoped that the prediction model can produce certain social and economic benefits in clinical practice. In addition, when comparing with other clinical studies on DR, we found some controversies, such as the impact of sex and body mass index on DR, which opened up a new direction for future research.

FOOTNOTES

Author contributions: Zhou YY contributed to the conception and design, acquisition of data or analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content; Yang Y and Zhou TC were responsible for supervision, project administration, and funding acquisition; Chen N and Zhou GZ were responsible for literature and formal analysis; Wang JR, Bai CF, Long R, Xiong YX, Zhou HJ, and Li XD were responsible for patient recruitment and clinical data curation; all authors gave final approval of the version to be published.

Supported by the Natural Science Foundation of China, No. 82160159; Natural Science Foundation of Yunnan Province, No. 202101AY070001-199; Scientific Research Fund of Yunnan Education Department, No. 2021J0303; and Postgraduate Innovation Fund of Kunming Medical University, No. 2020D009.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Affiliated Hospital of Yunnan University (Approval No. 2021062).

Informed consent statement: Written informed consent was obtained from all participants.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2022 December 15; 13(12): 1001-1183

Published by Baishideng Publishing Group Inc

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NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Jian-Bo Xiao, Michael Horowitz

EDITORIAL BOARD MEMBERS
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PUBLICATION DATE
December 15, 2022

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World J Diabetes 2022 December 15; 13(12): 1001-1013

DOI: 10.4239/wjd.v13.i12.1001

ISSN 1948-9358 (online)

REVIEW

Non-coding RNAs: Role in diabetic foot and wound healing

Yi-Bo Tang, Muhuza Marie Parfaite Uwimana, Shu-Qi Zhu, Li-Xia Zhang, Qi Wu, Zhao-Xia Liang

Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Li H, China; Mostafavinia A, Iran; Terabe Y, Japan

Received: August 21, 2022
Peer-review started: August 21, 2022
First decision: October 21, 2022
Revised: October 26, 2022
Accepted: November 18, 2022
Article in press: November 18, 2022
Published online: December 15, 2022

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Abstract

Diabetic foot ulcer (DFU) and poor wound healing are chronic complications in patients with diabetes. The increasing incidence of DFU has resulted in huge pressure worldwide. Diagnosing and treating this condition are therefore of great importance to control morbidity and improve prognosis. Finding new markers with potential diagnostic and therapeutic utility in DFU has gathered increasing interest. Wound healing is a process divided into three stages: Inflammation, proliferation, and regeneration. Non-coding RNAs (ncRNAs), which are small protected molecules transcribed from the genome without protein translation function, have emerged as important regulators of diabetes complications. The deregulation of ncRNAs may be linked to accelerated DFU development and delayed wound healing. Moreover, ncRNAs can be used for therapeutic purposes in diabetic wound healing. Herein, we summarize the role of microRNAs, long ncRNAs, and circular RNAs in diverse stages of DFU wound healing and their potential use as novel therapeutic targets.

Key Words: Diabetic foot ulcer; Wound healing; MicroRNA; Long non-coding RNAs; Circular RNAs; Inflammation; Proliferation; Regeneration

Core Tip: Non-coding RNAs (ncRNAs) have emerged as important regulators of diabetic foot and wound healing. NcRNAs can be used for therapeutic purposes in diabetic wound healing. In this study, we summarize the roles of microRNAs, long ncRNAs, and circular RNAs in diverse stages of diabetic foot ulcer wound healing and their potential use as novel therapeutic targets.

Citation: Tang YB, Uwimana MMP, Zhu SQ, Zhang LX, Wu Q, Liang ZX. Non-coding RNAs: Role in diabetic foot and wound healing. *World J Diabetes* 2022; 13(12): 1001-1013
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1001.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1001

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease that is rapidly increasing worldwide. DM is a global public health burden with a negative impact on global health and socioeconomic development. Chronic hyperglycemia causes blood vessel inflammation, which leads to macroangiopathy and microangiopathy, particularly diabetic foot ulcer (DFU) and delayed wound healing. Delayed healing of chronic ulcer wounds in patients with diabetes is due to neuropathy, microangiopathy, and immune system dysfunction[1,2]. One of the leading causes of death in patients with diabetes is lower extremity amputation, which accounts for approximately 15% of DFU cases[3]. Different functional and structural microvascular changes in patients with diabetes increase the vulnerability of the skin and contribute to impaired wound healing[4]. DFU contributes to physical and psychological problems that hinder the health economy immensely. Conventional DFU treatments have an inefficient impact on reduction of the amputation rate; thus, a more efficient treatment is needed. Therefore, a better understanding of the molecular mechanisms and biomolecules involved in DFU development is necessary to provide better therapeutic options for wound healing.

Non-coding RNAs (ncRNAs) are potential novel biomarkers transcribed from the genome without protein translation function but can still perform specific biological functions. NcRNAs can be divided into two categories depending on the length of nucleotides; short-stranded RNAs or microRNAs (miRNAs) which are less than 200 nucleotides in length, and long ncRNAs (lncRNAs) which are greater than 200 nucleotides in length. Emerging evidence suggests that ncRNAs have an important regulatory role in various metabolic diseases, such as DM, based on the development of microarray and high-throughput sequencing[5]. In addition, some lncRNAs are covalently bound to the 3′-5′ end, forming circular RNAs (circRNAs)[6]. NcRNAs can be protected from the effects of RNA enzyme activity, temperature changes, and extreme pH values by binding to proteins or being packaged into extracellular vesicles. In this way, ncRNAs can maintain a stable state in the extracellular environment and can be used as a potential biomarker for diagnosing and treating diseases[7-9]. NcRNAs regulate cellular chromatin rearrangements, histone modifications, variable splicing gene modifications, or gene expression; mediate different biological processes; and ultimately influence the development of certain diseases[10]. Exosome-cargoed ncRNAs have been reported as pivotal regulators of angiogenesis during wound closure[11]. This background confers the possible treatment of delayed wound healing using ncRNAs. In this study, we summarize the role and mechanism of miRNAs, lncRNAs, and circRNAs in the pathogenesis and process of wound healing in DFU and the research progress of ncRNAs in cell therapy.

WOUND HEALING PROCESS

Wound healing is a complex and highly regulated process divided into three phases: Inflammation, proliferation, and regeneration[12]. Diabetic wound healing is widely associated with different cellular components and the extracellular matrix (ECM) in different parts of the skin[13]. The main effector cells in the inflammatory phase are macrophages. When normal skin is damaged, macrophages polarize to M1 phenotype, producing pro-inflammatory cytokines and stimulating endothelial cells and fibroblasts to release reactive oxygen species (ROS) to remove bacteria and debris from wounds. The subsequent shift to the M2 phenotype is correlated with remission of the inflammatory response and wound remodeling[14,15]. In diabetic wounds, the persistence of the M1 phenotype and the inability to subsequently polarize to the M2 phenotype are the key components delaying wound healing. Angiogenesis is the main basis of the proliferative phase of wound healing, cell proliferation, migration, and differentiation[14]. The integrity of the endothelial cell structure plays a very important role in maintaining normal blood circulation in the body. In healthy tissues, endothelial cells are in a quiescent phase. In diabetic patients, wound healing is slowed by decreased angiogenic growth factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and hypoxia-inducible factor (HIF)-1α[16-18]. An unfavorable diabetic wound environment promotes the dysregulation of key signaling pathways, such as Notch and PI3K/AKT/eNOS[19,20]. The regenerative phase of wound healing includes re-epithelialization and ECM remodeling. Reduced blood flow restricts the migration of leukocytes, keratinocytes, fibroblasts, and endothelial cells to the wound, which is detrimental to wound healing[21]. Fibroblasts proliferate and secrete ECM components, such as collagen fibers, which provide supportive structures for cell proliferation and migration to restore skin tissue function and

integrity to maintain tissue elasticity and strength[22]. DFUs have collagen degeneration and deformation and reduced fibroblasts in the proliferation and migration stages[23]. Keratinocytes are the main constituent cells of the epidermis involved in skin wound healing through migration, proliferation, and differentiation[24]. In addition, epithelial-to-mesenchymal transition (EMT) plays a crucial role in DFU regeneration and wound healing[25]. Many studies have shown that ncRNAs regulate EMT involved in DFU and wound healing[26,27]. The wound healing process is shown in Figure 1.

MIRNAS

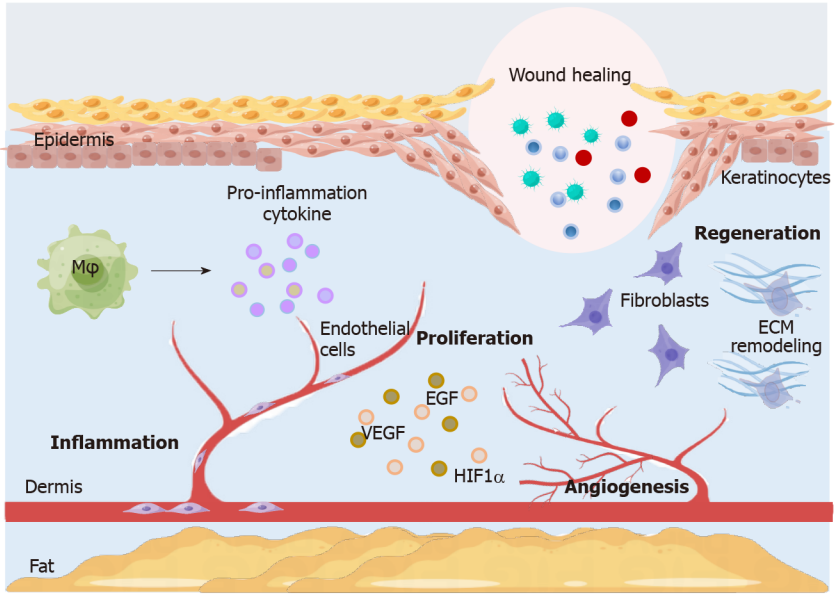
MiRNAs are a class of endogenous small ncRNAs with a molecular length of 18–25 nucleotides that regulate gene and/or protein expression at the post-transcriptional level by specifically binding to the 3′-untranslated region of downstream target miRNAs. The increased prevalence of diabetes has prompted increasing research into the mechanisms of miRNAs as therapeutic targets in DFU and wound healing. A study showed that low miR-24 expression is an independent risk factor for DFU in multifactorial logistic regression analysis[28]. Furthermore, low miR-24 expression is negatively correlated with fasting blood glucose and glycated hemoglobin and positively correlated with inflammatory markers[28-30]. MiRNAs have been associated with DFU progression and severity; specific miRNAs, such as miR-26, increase DFU severity[31], whereas other miRNAs, such as miR-129 and miR-335, improve wound healing[26].

Inflammation

MiR-217 belongs to the group that increases DFU severity. A study showed that a dual luciferase reporter gene assay confirmed HIF-1α as a direct target gene of miR-217. MiR-217 expression was upregulated whereas HIF-1α/VEGF expression was downregulated in patients with DFU and in the serum of rats with DFU compared with DM and healthy controls[32]. MiR-23c is upregulated in the peripheral blood and wound tissue in DFU, targeting stromal cell-derived factor-1α and inhibiting wound angiogenesis by recruiting inflammatory cells, such as macrophages[33]. In a mouse DFU model, miR-497 expression was downregulated, which considerably increased the expression of pro-inflammatory factors, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, resulting in a prolonged inflammatory phase of wound healing[34]. MiR-155 regulates insulin sensitivity and blood glucose levels in mice[35]. MiR-155 is markedly upregulated in diabetic skin[36]. MiR-155 has pro-inflammatory effects; thus, miR-155 inhibition leads to reduced inflammation, increased macrophage M2 polarization, reduced IL-1β and TNF-α levels, more regular collagen fiber alignment, and faster diabetic wound healing[37-39]. MiR-217, miR-497, and miR-155 are effector molecules in the inflammatory phase of diabetic wound healing; however, a further exploration of their mechanisms might improve wound healing during the inflammatory phase.

Proliferation

Angiogenesis is an essential step in the proliferative phase associated with DFU prognosis and wound healing. Recent studies have focused on the mechanisms and applications of miRNAs in regulating angiogenesis during the proliferative phase[40-42]. A maggot therapeutic approach study found that miR-18a/19a is markedly upregulated and thrombospondin-1 (TSP-1) expression is downregulated in DFU wounds as a result of impaired angiogenesis. The target activation of miR-18a/19a transcript levels and the regulation of TSP-1 expression may be a novel strategy for DFU treatment[40]. MiR-15a-3p is upregulated in the blood exosomes of patients with diabetes[41]. *In vivo* and *in vitro* experiments showed that exosomes with low miR-15a-3p expression inhibited diabetic wound healing. By contrast, knockdown of circulating exosomal miR-15a-3p expression may accelerate wound healing through the activation of NADPH oxidase (NOX) 5 and increase ROS release[41]. NOX activates redox signaling pathways and promotes angiogenesis[43]. Phosphatase and tensin homolog (PTEN) expression is regulated by blood glucose concentrations, is mainly found in epithelial cells, and activates signaling cascades that affect angiogenesis[44]. MiR-152-3p is an upstream negative regulator of PTEN upregulated in diabetic wounds; hence, inhibiting the angiogenic function of PTEN leads to delayed wound healing[45]. MiR-195-5p and miR-205-5p carried by extracellular vesicles in DFU wound fluid negatively regulate angiogenesis and wound healing in DFU[42]. Increased miR-133b expression induces downregulation of EGF receptor (EGFR), affecting endothelial cell proliferation and angiogenesis in all diabetic wounds. *In vitro* experiments showed that miR-133b downregulation in human umbilical vein endothelial cells partially reverses impaired angiogenesis[46]. These findings imply that miR-133b negatively regulates angiogenesis during the proliferative phase of wound healing. Huang *et al*[47] found that miR-489-3p downregulation increases sirtuin (SIRT) 1 expression, promotes the PI3K/AKT/eNOS signaling pathway, improves cellular antioxidant capacity, and alleviates DFU. MiR-199a-5p has an important role in the development of diabetes and its complications[48,49]. Moreover, miR-199a-5p promotes apoptosis and ROS production within pancreatic β-cells in type 2 DM (T2DM)[50]. MiR-199a-5p sponge-adsorbed to hsa-circ-006040 inhibits macrophage-mediated inflammatory responses in type 1 DM (T1DM)[48]. Wang *et al*[49] found that downregulating miR-199a-3p in



DOI: 10.4239/wjd.v13.i12.1001

Figure 1 A diagram of the diabetic foot wound healing process. In the inflammation phase, macrophages produce pro-inflammatory cytokines. In the proliferation phase, angiogenic growth factors promote angiogenesis by stimulating endothelial cell proliferation and migration. In the regeneration phase, fibroblasts proliferate and secrete extracellular matrix components to provide supportive structures for cell proliferation and migration to restore skin tissue function. Mφ: Macrophages; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; HIF-1α: Hypoxia-inducible factor 1 α; ECM: Extracellular matrix.

endothelial cells alleviates inhibition of the target VEGFA and Rho-related kinase 1, rescuing the cellular damage induced by high glucose and restoring angiogenic function. Therefore, these findings suggest that regulating miRNA expression during the proliferative phase of wound healing has great potential in DFU treatment and wound repair.

Regeneration

Recently, Moura *et al*[36] also found that the local inhibition of miR-155 in diabetic wounds increased the expression of its target, fibroblast growth factor (FGF) 7, which sequentially increased re-epithelialization and accelerated wound healing[36,51]. Yuan *et al*[52] found that miR-203 upregulation in DFU tissues may inhibit the EMT process and delay wound healing in a rat DFU model. On the contrary, miR-203 knockdown promoted wound healing by activating the target gene, IL-8, and IL-8/AKT downstream pathways. High miR-203 expression reduces keratinocyte proliferation and migration, partially explaining the development of DFU into chronic refractory wounds[52]. On the contrary, recent studies have found that negative pressure wound therapy can reverse the inhibition of keratinocytes as a result of high levels of miR-203 by reducing miR-203 in the peripheral blood and wound tissue and upregulating p63 expression[53]. Sprouty homolog (SPRY) 1, an antagonist of the FGF pathway, is expressed in fibroblasts, and its downregulation plays an important role in wound healing[54,55]. MiR-21-3p is downregulated in diabetic patients compared with healthy controls and in fibroblasts stimulated with D-glucose compared with control fibroblasts[56]. Enhanced miR-21-3p expression may inhibit SPRY1, stimulate fibroblast proliferation and migration, and accelerate wound healing[42]. MiR-146a is downregulated in DFU wound tissue. Bioinformatics analysis revealed that A-kinase-anchoring protein 12 (AKAP12) and Toll-like receptor 4 (TLR4) are the target genes of miR-146a. Peng *et al*[57] showed that miR-146a activates in the inflammatory phase of diabetic wound healing by inhibiting the TLR4/nuclear factor-kappaB axis involved in macrophage M2 polarization. In addition, Zhang *et al*[58] constructed an *in vitro* DFU model using human keratinocyte-derived HaCaT cells and demonstrated that miR-146a is activated during the tissue regeneration phase. *In vivo* and *ex vivo* results showed that miR-146a overexpression inhibited the angiogenic regulator AKAP12, activated the HIF-1α/Wnt3α/β-catenin signaling pathway, and promoted cell proliferation and migration[57]. MiRNAs have regulatory effects on a wide range of cells involved in tissue remodeling during the regeneration phase. MiRNAs are the most studied ncRNAs and act in various periods of DFU and wound healing, respectively, or continuously. We summarized some of the considerably altered miRNAs in diabetic patients as shown in Table 1. Notably, most of these pooled miRNAs have not been reported to have a clear therapeutic role in DFU and should therefore be evaluated in future studies.

Table 1 Micro-RNAs in diabetic foot and wound healing

Name	Expression	Animal	Target gene	Pathway	Phase	Ref.
miRNA-217	Up	Mouse	*HIF-1α*	VEGF	Inflammation	Lin *et al*[32], 2019
miRNA-23c	Up	/	*SDF-1α*	SDF-1α/CXCL12	Inflammation	Amin *et al*[33], 2020
miRNA-497	Down	Mouse	*IL-1β, IL-6, TNF-α*	NF-κB	Inflammation	Ban *et al*[34], 2020
miRNA-155	Up	Mouse	*FGF7*	/	Inflammation/regeneration	Moura *et al*[36], 2019; Gondaliya *et al*[51], 2022
miRNA-18a/19a	Up	/	*TSP-1*	/	Proliferation	Wang *et al*[40], 2020
miRNA-15a-3p	Up	Mouse	*NOX5*	ROS	Proliferation	Xiong *et al*[41], 2020
miRNA-152-3p	Up	Mouse	*PTEN*	/	Proliferation	Xu *et al*[45], 2020
miRNA-133b	Up	Mouse	*EGFR*	/	Proliferation	Zhong *et al*[46], 2021
miRNA-195-5p	Up	Rat	*VEGFA*	/	Proliferation	Liu *et al*[42], 2021
miRNA-205-5p	Up	Rat	*VEGFA*	/	Proliferation	Liu *et al*[42], 2021
miRNA-199a-5p	Up	Rat	*VEGFA, ROCK1*	/	Proliferation	Wang *et al*[49], 2022
miRNA-203	Up	Rat	*IL-8*	AKT	Regeneration	Yuan *et al*[52], 2019
		/	*p63*	/	Regeneration	Liu *et al*[53], 2022
miR-489-3p	Up	Rat	*SIRT1*	PI3K/AKT/eNOS	Regeneration	Huang *et al*[47], 2022
miRNA-21-3p	Down	Mouse	*SPRY1*	FGF	Regeneration	Wu *et al*[56], 2020
miRNA-146a	Down	/	*AKAP12*	Wnt/β-catenin	Regeneration	Peng *et al*[57], 2022
		/	*TLR4*	NF-κB	Inflammation	Zhang *et al*[58], 2022

HIF-1α: Hypoxia-inducible factor 1 α; VEGF: Vascular endothelial growth factor; SDF-1α: Stromal cell-derived factor-1α; IL: Interleukin; TNF: Tumor necrosis factor; FGF7: Fibroblast growth factor 7; TSP-1: Thrombospondin-1; NOX5: NADPH oxidase 5; ROS: Reactive oxygen species; PTEN: Phosphatase and tensin homolog; EGFR: Epidermal growth factor receptor; ROCK1: Rho-related kinase 1; SIRT1: Sirtuin 1; SPRY1: Sprouty homolog 1; AKAP12: A-kinase-anchoring protein 12; TLR4: Toll-like receptor 4; NF-κB: Nuclear factor-kappaB; PI3K: Phosphoinositide 3-kinase; eNOS: Endothelial nitric oxide synthase.

LNCRNAS

LncRNAs are located in highly conserved genomic regions with spatially and temporally tightly regulated expression and dysregulated expression profiles as important markers of altered disease or developmental status. The main mechanism and function of lncRNAs are to act as competing endogenous RNAs (ceRNAs) for miRNAs, which interact with mRNA target base pairs to control various signaling pathways[59]. Another mechanism is by interacting with RNA-binding proteins[60]. Increasing evidence shows that lncRNAs play an important role in diabetic complications. LncRNA 3632454L22RiK can promote corneal epithelial wound healing in diabetic mice by sponging miR-181a-5p[61]. The regulatory role of lncRNA MIAT in diabetic cardiomyopathy has also been demonstrated [62]. These findings indicate an increased awareness of lncRNAs in diabetic complications.

Inflammation

The mechanism of lncRNAs in the inflammatory phase lacks enough evidence. LncRNA growth arrest specific 5 (GAS5) has been identified as a tumor suppressor that inhibits cell proliferation and promotes apoptosis[63]. GAS5 expression was markedly elevated in DFU wounds[64]. GAS5 promotes the M1 phenotypic polarization of macrophages through the upregulation of signal transducer and activator of transcription 1 (STAT1), leading to prolonged inflammatory phase and delayed wound remodeling and closure[64]. STAT1 signaling is exactly the central pathway that controls M1-M2 polarization in macrophages. Reduced GAS5 levels in wounds appear to promote healing by facilitating the conversion of M1 macrophages to M2 macrophages. Thus, targeting lncRNA GAS5 may contribute to efficient therapeutic interventions for impaired wound healing in diabetes.

Proliferation

GAS5 regulates the inflammatory process of wound healing and plays a part in the proliferative phase. During the proliferative phase, GAS5 activates the HIF-1α/VEGF pathway by binding to TATA box-binding protein associated factor 15, stimulating endothelial cell proliferation and angiogenesis and leading to accelerated DFU wound healing[65]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a relatively well-studied transcript among lncRNAs. The role of MALAT1 has been reported in a variety of diseases, including renal tumors, osteosarcoma, and gestational diabetes[66-68]. MALAT1 protects endothelial cells from oxidative stress injury by activating the nuclear factor erythroid-2-related factor 2 (Nrf2) pathway. MALAT1 is markedly reduced in DFU-infected tissues, leading to insufficient HIF-1α/VEGF activation and impeding angiogenesis[69]. The exogenous uptake of exosome lnc01435 by vascular endothelial cells alters the subcellular localization of transcription factor yin yang 1 (YY1) and synergistically upregulates histone deacetylase (HDAC) 8 expression with YY1. HDACs are important components of the NOTCH signaling pathway with negatively regulated expression levels and thus affect endothelial cell function and angiogenesis[70,71]. In summary, targeting GAS5, MALAT1, and lnc01435 may help develop new therapeutic strategies to treat DFUs.

Regeneration

LncRNA H19, located on chromosome 11, exhibits negative regulation of diabetic wound healing. LncRNA H19 acts as a sponge for miR-29b and competitively represses miR-29b expression; therefore, it upregulates fibrillin 1 (FBN1), activates the transforming growth factor-β/Smad signaling pathway, and promotes ECM accumulation[72]. Connective tissue growth factor (CTGF) is a matricellular protein from the Cyr61/CTGF/Nov protein family, which interacts with ECM protein to mediate external signal transduction into cells through many subtypes of integrin receptors[73]. During the proliferative phase of diabetic wound healing, lncRNA H19 recruits the transcription factor SRF to the CTGF promoter region, activating CTGF and its downstream MAPK signaling pathway to accelerate fibroblast proliferation and wound healing[74]. These findings elaborate lncRNA H19 as a regulator in the regenerative phase of wound healing. A novel lncRNA MRAK052872, named lnc-upregulated in diabetic skin (URIDS), is involved in the mechanism of DFU wound healing. Lnc-URIDS is highly expressed in diabetic skin and dermal fibroblasts treated with advanced glycosylation end products. Lnc-URIDS binds to procollagen-lysine and 2-oxoglutarate 5-dioxygenase 1 (plod1), decreases plod1 protein stability, and leads to dysregulated collagen deposition and delayed wound healing[27]. LncRNA cancer susceptibility candidate 2 (CASC2) was originally discovered in an endometrial cancer study and is located on human chromosome 10q26[75]. Furthermore, CASC2 overexpression inhibited fibroblast migration and proliferation, suppressed apoptosis, and facilitated wound healing, especially in DFU mice. By contrast, miR-155 overexpression inhibited the function of CASC2[75]. Another study showed that HIF-1α inhibition reversed the effects of miR-155 downregulation on fibroblasts[76]. Evidently, lncRNAs have a considerable regulatory role in cellular functions during re-epithelialization and remodeling.

The mechanisms by which lncRNAs cause DFU and delayed wound healing are atypical inflammatory responses, impaired angiogenesis, impaired and abnormal ECM accumulation, and epithelial processes that regulate wound healing. The lncRNAs in DFU and delayed wound healing are listed in Table 2. These findings provide new information for the clinical treatment of diabetic chronic non-healing wounds.

CIRCRNAS

CircRNAs are a unique type of ncRNA derived from exons, introns, or intergenic regions that are covalently linked to produce a closed loop structure in the absence of 50 caps and 30 tails. CircRNAs are conserved among species owing to their resistance properties to RNase R. CircRNAs are involved in a wide range of biological processes, such as transcription and mRNA splicing, RNA decay, and RNA translation; the dysregulation of circRNAs leads to abnormal cellular functions and human diseases[77,78]. CircRNAs can also act as a miRNA sponge to inhibit miRNA function, which plays a crucial role in the pathogenesis of diabetes and its vascular complications[79]. Circ-PNPT1 and has_circ_0046060 promote the development of gestational DM by regulating trophoblast cell function or causing insulin resistance[80,81]. Circ-ITCH improved renal inflammation and fibrosis in diabetic mice by regulating the miR-33a-5p/SIRT6 axis[82]. CircRNAs are closely related to the development of diabetes and its complications. Studies on the role and mechanism of circRNAs in DFU and delayed wound healing are relatively few. Existing studies evaluated the regulatory role of circRNAs on angiogenesis and re-epithelialization.

CircRNAs protein kinase, DNA-activated, catalytic subunit (circ_PRKDC, has-circ-0084443) is involved in the promotion of keratinocyte proliferation and the suppression of keratinocyte migration during wound healing[83]. Circ_PRKDC negatively regulates keratinocyte migration *via* the EGFR pathway, impeding re-epithelialization and angiogenesis[84]. However, circ_PRKDC knockdown promotes epidermal keratinocyte migration *via* the miR-31/FBN1 axis[83]. This finding shows that

Table 2 Long non-coding RNAs in diabetic foot and wound healing

Name	Expression	Sponge	Animal	Target gene	Pathway	Phase	Ref.
GAS5	Up	/	Mouse	*STAT1*	/	Inflammation	Hu *et al*[64], 2020
		/	Mouse	*TAF15*	HIF-1α/VEGF	Proliferation	Peng *et al*[65], 2021
MALAT1	Down	/	/	*HIF-1α/Nrf2*		Proliferation	Jayasuriya *et al*[69], 2020
Lnc01435	Up	/	Mouse	*YY1, HDACs*	Notch	Proliferation	Fu *et al*[70], 2022
H19	Up	miRNA-29b	Mouse	*FBN1*	TGF-β/Smad	Regeneration	Li *et al*[72], 2021
	Up	/	Rat	*CTGF, SRF*	MAPK	Regeneration	Li *et al*[74], 2020
URIDS	Up	/	Rat	*Plod1*	VEGF/TGF-β	Regeneration	Hu *et al*[27], 2020
CASC2	Down	miR-155	Mouse	*HIF-1α*	/	Regeneration	He *et al*[76], 2022

GAS5: Growth arrest specific 5; STAT1: Signal transducer and activator of transcription 1; TAF15: TATA box-binding protein associated factor 15; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; YY1: Yin yang 1; HDAC8: Histone deacetylase 8; FBN1: Fibrillin 1; CTGF: Connective tissue growth factor; SRF: Serum response factor; URIDS: Upregulated in diabetic skin; Plod1: Procollagen-lysine and 2-oxoglutarate 5-dioxygenase 1; CASC2: Cancer susceptibility candidate 2; HIF-1α: Hypoxia-inducible factor 1 α; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; Nrf2: Nuclear factor erythroid 2-related factor 2.

circ_PRKDC has therapeutic potential for skin wound healing. Shang *et al*[85] evaluated the effect of circ-Klhl8 in epithelial progenitor cells (EPCs) on diabetic wound closure by establishing an *in vivo* mouse model of total skin defect and found that circ-Klhl8 overexpression increases the therapeutic effect of EPCs to promote diabetic wound healing by targeting the miR-212-3p/SIRT5 axis. Altered circRNA expression can affect disease progression and wound healing in DFU (Table 3). Studies on circRNAs in various stages of DFU and wound healing are few and prompted the need for further research on functional circRNAs in the future to identify limitations in DFU treatment.

NCRNAS IN CELL THERAPY

The standard treatment for DFUs includes optimizing blood flow, debridement, infection control, and offloading. In standard treatment, only 50% of patients heal within 20 wk and 50% relapse within 18 mo; thus, efficient treatment for DFUs are urgently needed[86]. Cell-based DFU therapy is a new treatment intervention therapy studied in the last few years. Stem cells can affect ulcer healing through various pathophysiological processes, such as stimulating tissue repair, increasing ECM synthesis, and promoting angiogenesis in ischemic tissues[87]. Soluble factors and extracellular vesicles secreted by stem cells are active factors in diabetic wound healing. Extracellular vesicles from mesenchymal stem cells (MSCs) are considered an alternative treatment for immune disorders, including diabetes. Emerging evidence suggests that MSC-derived exosomes applied to the wound surface can promote angiogenesis and tissue repair[88]. MSC regenerative therapy is a novel tissue regeneration modality that accelerates wound healing in DFU and identifies patients at high risk of amputation[89]. Adipose-derived stem cells (ADSCs) have become an alternative to cell therapy owing to their abundance, subcutaneous location, easy accessibility, and longer culture time than bone marrow mesenchymal cells (BMSCs) and thus exert greater proliferation and differentiation capacity. Previous studies found that ADSC transplantation can promote foot wound healing in diabetic rats whereas stem cell transplantation may have clinical application in DFU treatment[90]. EPCs are the precursor cells of vascular endothelial cells that can be directed to the site of ischemic injury and form new vessels through proliferation and differentiation to promote wound healing[91]. Cell-derived exosomes loaded with ncRNAs have a therapeutic effect on refractory DFUs.

Gondaliya *et al*[51] revealed the therapeutic potential of miR-155 inhibitor-loaded MSC-derived exosomes in diabetic wound healing and demonstrated that wrapping miRNA and antibiotics in MSC-derived exosomes improved the management of chronic, non-healing diabetic wounds. Studies found that miR-129 may promote diabetic wound healing by balancing ECM synthesis and degradation through the inhibition of Sp1-mediated matrix metalloproteinase 9 expression[26]. A recent study also showed that miR-129 loaded in MSC-derived extracellular vesicles promoted wound healing *via* the downregulation of tumor necrosis factor receptor-associated factor 6[92]. Evidently, miR-129 is an important regulator of the proliferative and regenerative phases of wound healing and may be a biologically active molecule in MSC for DFU treatment. Xu *et al*[93] showed that miR-221-3p in EPC-derived exosomes accelerated skin wound healing in normal and diabetic mouse models. The latest study further demonstrated the mechanism of miR-221-3p in diabetic wound treatment[94]. MiR-221-3p

Table 3 Circular RNAs in diabetic foot and wound healing

Name	Expression	Sponge	Animal	Target gene	Phase	Ref.
Circ_PRKDC	Up	/	/	*EGFR*	Proliferation	Wang *et al*[84], 2020
	Up	miR-31	/	*FBN1*	Proliferation	Han *et al*[83], 2021
Circ_Klhl8	Down	miR-212-3p	Mouse	*SIRT5*	Proliferation	Shang *et al*[85], 2021

Circ_PRKDC: Circular RNA protein kinase, DNA-activated, catalytic subunit; SIRT5: Sirtuin 5; FBN1: Fibrillin 1; EGFR: Epidermal growth factor receptor.

overexpression may inhibit the anti-angiogenic function of its direct targeted homeodomain-interacting protein kinase 2 (HIPK2) and promote endothelial cell proliferation[94].

Li *et al*[95] showed that the MSC-derived exosomal lncRNA, lncRNA H19, causes fibroblast inflammation and apoptosis by disrupting miR-152-3p-mediated PTEN inhibition, leading to a stimulated wound-healing process in DFU. MSCs have demonstrated a therapeutic effect in DFU by generating pro-angiogenesis factors, such as VEGF. Recent research shows that genetically modified MSCs have been used in therapy, and the depletion of miR-205-5p in human MSCs promotes VEGF-mediated therapeutic effects in DFU[96,97]. LncRNA MALAT1 is a ceRNA for miR205-5p but has a low expression in human MSCs. Ectopic MALAT1 expression in human MSCs considerably decreased miR-205-5p levels, resulting in the upregulation of VEGF production and improved *in vitro* endothelial cell tube formation. In an immunodeficient NOD/SCID mouse model of diabetic foot (DF), the transplantation of human miR-205p-depleted MSCs resulted in better therapeutic effects on DF recovery than control MSCs. Moreover, MALAT1-expressing MSCs showed even better therapeutic effects on DF recovery than miR-205-5p-depleted MSCs. This difference in DF recovery was associated with the levels of on-site vascularization. Overall, MALAT1 functions as a sponge RNA for miR-205-5p to increase the therapeutic effects of MSCs on DF[97]. As mentioned above, miR-205-5p is an anti-angiogenic factor that inhibits VEGFA expression at the post-transcriptional level in MSCs, and the inhibition of its expression leads to angiogenesis and considerably improves the therapeutic effect of MSCs on diabetic wounds[97,98]. BMSC-derived exosomes can encapsulate lncRNA Kruppel-like factor 3 antisense RNA 1 (KLF3-AS1); adequately promote vascular endothelial cell proliferation, migration, and tube formation; and inhibit high glucose-induced apoptosis[99]. Diabetic wound healing by lncRNA KLF3-AS1 encapsulated by MSC-derived exosomes was achieved by downregulating miR-383 and upregulating its target, VEGFA[99].

High-throughput sequencing revealed an abnormally reduced expression of mmu_circ_0000250 in diabetic mice[100]. Exosomes from mmu_circ_0000250-modified ADSCs promote wound healing in diabetic mice through the induction of miR-128-3p/SIRT1-mediated autophagy[100]. In the study by Shi *et al*[100], the exosomes of ADSCs exerted therapeutic effects by restoring vascular endothelial cell function under high-glucose conditions. Circ-0000250 expression may increase the effectiveness of exosome therapy. Circ_ARHGAP12 is a cyclic molecule that inhibits high glucose-induced cell apoptosis by enhancing cellular autophagy[101]. Circ_ARHGAP12 was able to directly interact with miR-301b-3p and subsequently stimulate miRNAs to regulate the expression of ATG16L1 and ULK2, the target genes of miR-301b-3p, as well as downstream signaling pathways[101]. These findings propose a prospective therapeutic strategy of targeting circ_ARHGAP12 in MSCs to promote diabetic wound healing. Recent studies have found that circRNAs HIPK three (circHIPK3)-rich exosomes derived from human umbilical cord-derived MSCs have promising therapeutic effects in DFU. Exosomal circHIPK3 significantly promotes revascularization and wound healing by sponging to miR-20b-5p and upregulating the Nrf2/VEGFA axis[102]. Some ncRNAs for the cell therapy of DFU are shown in Table 4. NcRNAs and vector exosomes are effector molecules with great potential among the cellular therapeutic approaches for DFU and are expected to be of clinical use in the near future.

CONCLUSION

This study summarized the role and intrinsic mechanisms of ncRNAs in diabetic wound healing and provided more potential targets for future studies on wound healing in patients with diabetes. NcRNAs are regulatory molecules that modify many physiological processes and aspects of human diseases. The inflammation, proliferation, and regeneration phases of diabetic wound healing overlap, and ncRNAs are biologically active during all three phases. NcRNAs have a crucial role in the pathogenesis and impairment of wound healing in patients with diabetes. NcRNAs activate certain signaling pathways by downregulating or upregulating certain genes. Some of these molecules may provide valuable information in the clinical setting and serve as diagnostic or screening tools to predict high-risk DFUs and provide a basis for early prevention. These findings suggest that cell therapy using ncRNAs for DFU has great potential in the field of regenerative medicine.

Table 4 Non-coding RNAs in cell therapy

Name	Origin	Expression	Sponge	Target gene	Phase	Ref.
miRNA-155	MSC	Up	/	*FGF7*	Proliferation	Moura *et al*[36], 2019; Gondaliya *et al*[51], 2022
miR-129	MSC	Down	/	*TRAF6*	Proliferation	Hu *et al*[92], 2022
miRNA-221-3p	EPC	Down	/	*HIPK2*	Proliferation	Yu *et al*[94], 2022
LncRNA H19	MSC	Up	miRNA-152-3P	*PTEN*	Proliferation	Li *et al*[95], 2020
MALAT1	MSC	Down	miR-205-5p	*VEGF*	Proliferation	Zhu *et al*[97], 2019
Lnc KLF3-AS1	BMSC	Down	miR-383	*VEGFA*	Proliferation	Han *et al*[99], 2022
Circ_0000250	ADSC	Down	miR-128-3p	*SIRT1*	Proliferation	Shi *et al*[100], 2020
Circ_ARHGAP12	MSC	Down	miR-301b-3p	*ATG16L1, ULK2*	Proliferation	Meng *et al*[101], 2022
Circ HIPK3	MSC	Down	miR-20b-3p	*Nrf2/VEGFA*	Proliferation	Liang *et al*[102], 2022

MSC: Mesenchymal stem cells; FGF7: Fibroblast growth factor 7; TRAF6: Tumor necrosis factor receptor-associated factor 6; EPC: Epithelial progenitor cells; HIPK2: Homeodomain-interacting protein kinase 2; PTEN: Phosphatase and tensin homolog; SIRT1: Sirtuin 1; VEGF: Vascular endothelial growth factor; Lnc KLF3-AS1: LncRNA Kruppel-like factor 3 antisense RNA 1; BMSC: Bone marrow mesenchymal cells; ADSC: Adipose-derived stem cells; Circ HIPK3: Circular RNA homeodomain-interacting protein kinase three; Nrf2: Nuclear factor erythroid 2-related factor 2; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; Lnc: Long non-coding; miRNA: Micro RNA.

FOOTNOTES

Author contributions: Tang YB and Uwimana MMP contributed to the design and drafting of the work; Zhu SQ contributed to the acquisition and analysis of data; Zhang LX and Wu Q contributed to the revision of the paper; Liang ZX contributed to the study conception; and all authors read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 81974234.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1014-1034

DOI: 10.4239/wjd.v13.i12.1014

ISSN 1948-9358 (online)

REVIEW

Diabetic foot ulcer: Challenges and future

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Miranda C, Italy; Mostafavinia A, Iran; Mrozikiewicz-Rakowska B, Poland; Terabe Y, Japan

Received: August 19, 2022
Peer-review started: August 19, 2022
First decision: September 26, 2022
Revised: October 7, 2022
Accepted: November 28, 2022
Article in press: November 28, 2022
Published online: December 15, 2022

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Abstract

Diabetic foot ulcers (DFUs) have become one of the important causes of mortality and morbidity in patients with diabetes, and they are also a common cause of hospitalization, which places a heavy burden on patients and society. The prevention and treatment of DFUs requires multidisciplinary management. By controlling various risk factors, such as blood glucose levels, blood pressure, lipid levels and smoking cessation, local management of DFUs should be strengthened, such as debridement, dressing, revascularization, stem cell decompression and oxygen therapy. If necessary, systemic anti-infection treatment should be administered. We reviewed the progress in the clinical practice of treating DFUs in recent years, such as revascularization, wound repair, offloading, stem cell transplantation, and anti-infection treatment. We also summarized and prospectively analyzed some new technologies and measurements used in the treatment of DFUs and noted the future challenges and directions for the development of DFU treatments.

Key Words: Diabetic foot ulcer; Diabetic peripheral artery disease; Diabetic peripheral neuropathy

Core Tip: Diabetes foot ulcer has become one of the important causes of mortality and morbidity of diabetes patients, and it is also a common cause of hospitalization, which brings a heavy burden to patients and society. The prevention and treatment of diabetes foot ulcer needs multidisciplinary management. We reviewed the progress in the clinical practice of diabetes foot ulcer in recent years, such as revascularization, wound repair, offloading, stem cell transplantation, anti-infection treatment. We also summarized and prospected some new technologies and measurements in the treatment of diabetes foot ulcer, and pointed out the future challenge and development direction of diabetes foot ulcer.

Citation: Yang L, Rong GC, Wu QN. Diabetic foot ulcer: Challenges and future. *World J Diabetes* 2022; 13(12): 1014-1034
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1014.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1014

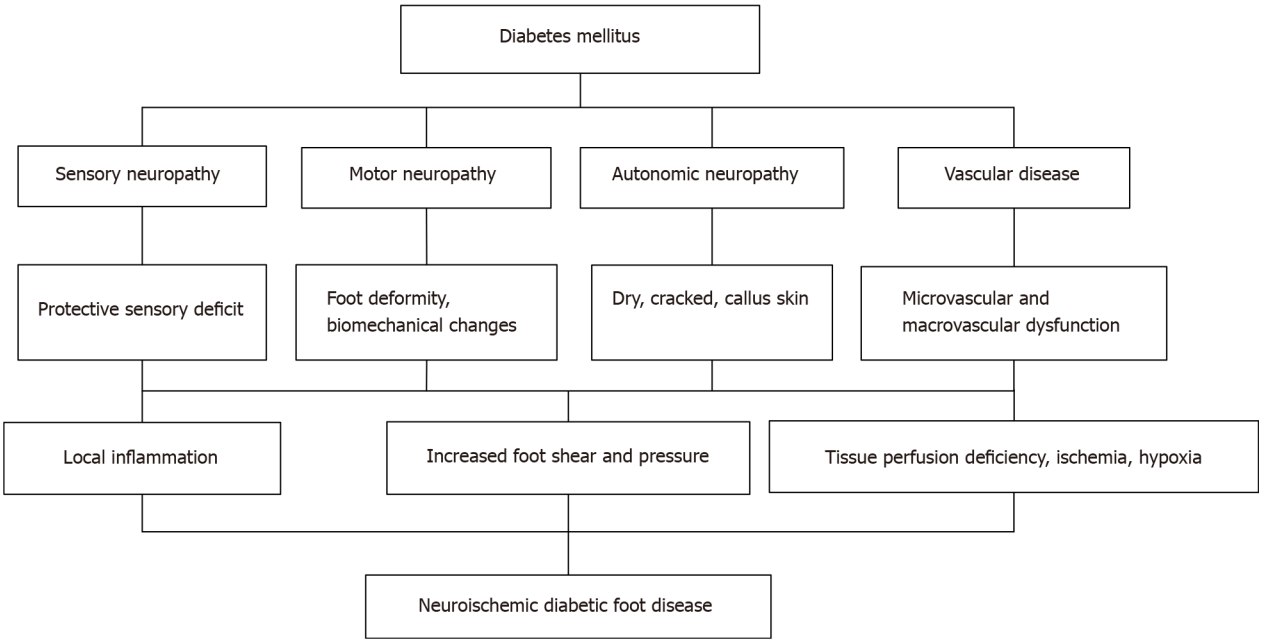
INTRODUCTION

The prevalence of diabetes is gradually increasing: the global prevalence was estimated to be 9.3% (463 million people) in 2019 and is expected to increase to 10.2% (570 million people) in 2030 and 10.9% (700 million people) in 2045[1]. The prevalence rate of diabetes in Chinese people older than 18 years of age is 11.2%[2]. Diabetic foot ulcer (DFU) is one of the most serious and dreaded complications of diabetes. A total of 10%-15% of patients with diabetes may experience foot ulcers[3]. At least half of all amputations occur in patients with diabetes, and the most common cause is DFU infection. In a large cohort study of patients with DFU and patients with diabetes in China, the annual ulcer incidence rate among diabetic patients was 8.1%, the annual new ulcer incidence rate among patients with DFU was 31.6%, the amputation rate among patients with DFU was 5.1%, and the annual mortality rates among patients with diabetes and patients with DFU were 2.8% and 14.4%, respectively, during the 1-year follow-up period[4]. DFU is the main cause of hospitalization, amputation, deterioration of quality of life and disability of patients, which imposes a heavy economic burden on the medical and health system, and its economic burden ranks tenth among all diseases[5]. Therefore, the management of DFUs is particularly important. This article mainly introduces the risk factors and treatment of DFUs.

RISK FACTORS FOR DFU

The World Health Organization and the International Diabetes Federation define DFU as a serious complication of diabetes, mainly due to foot tissue ulcers and wounds caused by hyperglycemia, diabetic peripheral vascular disease and/or diabetic peripheral neuropathy[1]. DFU results from multiple factors. The risk factors for DFU must be addressed to reduce the rates of foot ulcers and amputation (Figure 1).

Neuropathy is a common complication of diabetes that occurs in 50% of patients with type 2 diabetes. Neuropathy is an important cause of ulcers. Long-term hyperglycemia leads to peripheral nerve fiber damage. Distal sensor motor peripheral neuropathy is the most common type. It manifests as distal, symmetric, and length-dependent multiple neuropathy[6]. Usually, small nerve fibers are damaged earlier than larger nerve fibers[7]. The dysfunction of small-fiber nerves leads to sensory changes, such as sensory dullness, acupuncture sensations, numbness, burning sensations, abnormal pain and other clinical symptoms. Sensory defects, such as defects in pain perception and temperature perception, are clinically called protective sensory deficits. The loss of protective sensation leads to a loss of sensitivity to injury and stimulation of the lower limbs, thus leading to continuous unconscious trauma, which tends to form ulcers. Usually, diabetic ulcers are found when blood stains are observed on the socks and floor, which portends an untimely diagnosis and treatment of the ulcers and aggravates the disease. Compared with patients with diabetes presenting without neuropathy, patients with diabetes presenting protective sensory loss have a 7-fold increased risk of developing DFUs[8]. The autonomic nerves will also be damaged. Dysfunction of the peripheral sympathetic nerves may lead to reduced sweating, dry skin, cracking and an increased risk of calluses complicated with peripheral arterial disease, and the appearance of symptoms increased. In the absence of peripheral artery disease, the dorsal foot vein expands, the foot feels warm, and some edema occurs. This situation places the patient's foot at high risk of ulceration. Biomechanical changes occur in the early stage of diabetic neuropathy[9]. Motor neuropathy causes an imbalance of foot muscle tissue, muscle weakness and atrophy and changes the normal foot dynamics and pressure distribution, leading to the loss of joint stability and the development of foot deformities such as claw toe, hammer toe, horseshoe foot, Charcot's ankle, arch changes, and plantar aponeurosis[10]. The increased shear stress and friction force increase the risk of foot ulcers, and when these factors are combined with the loss of protective sensation, the risk of foot



DOI: 10.4239/wjd.v13.i12.1014

Figure 1 Pathogenesis of diabetic foot ulcer.

ulcers is higher.

Lower extremity arterial disease is an important risk factor for DFUs, resulting in an insufficient blood supply, hypercoagulability of the lower extremities, and serious limb ischemia[11]. The clinical manifestations are malnutrition, muscle atrophy, decreased skin temperature, pigmentation, weakened or absent limb artery pulsation, and even intermittent claudication, resting pain and ulceration of the lower limbs. Long-term ischemia and hypoxia render the areas prone to tissue ulcers through the action of external forces, particularly ulcers at compressed parts of the heel or metatarsophalangeal joint, which are prone to secondary infection. Patients with diabetes usually have lower-limb arterial disease and neuropathy, which lead to difficult healing of neuropathic and ischemic ulcers. In 50%-75% of cases, peripheral arterial lesions lead to wound occurrence or a failure to heal[12,13].

The age and course of diabetes also affect ulcer healing. The risk of ulcer and amputation increases two to four times with increasing age and a prolonged disease course. Repeated minor injuries caused by neuropathic foot pressure and inappropriate footwear may increase the risk of ulceration. One study indicated that the overall risk of injury in patients with diabetes was 2% per year, the risk for patients with diabetes neuropathy increased to 7.5%, the risk for patients with a previous ulcer history increased to 40%, and the risk further increased to approximately 60% after 3 years and reached 75% after 5 years [14]. If the patient has eye diseases such as retinopathy and cataracts, resulting in decreased vision, the risk of a foreign body stabbing the foot is high, and the risk of ulceration is also increased. Some studies have suggested that dialysis patients with diabetic nephropathy have a very high risk of foot ulcers, and dialysis treatment is an independent risk factor for foot ulcers[15].

TREATMENT OF DFU

The ultimate goal of DFU treatment is to achieve healing and prevent wound infection, amputation and reduced quality of life. It mainly includes glycemic control, management of peripheral artery disease (PAD), and wound management, among others.

Glycemic control

Glycated hemoglobin may be the best indicator to evaluate average blood glucose control. An HbA1c level ≥ 8% and fasting blood glucose level ≥ 7 mmol/L are associated with an increased risk of lower limb amputation in patients with DFUs[16]. Studies have recommended that the glycated hemoglobin level in patients with DFUs should be controlled at 7%-8%, which is helpful for ulcer healing and will not increase the mortality of patients[17]. In another study, the glycosylated hemoglobin level was related to the wound healing speed, which was more obvious in patients with neuropathy and lower-limb arterial disease[18]; however, the results were not repeated in another study[19]. However, an appropriate blood glucose level is undoubtedly beneficial to prevent and delay microvascular and macrovascular complications in patients with diabetes[20]. The ideal blood glucose control target is

reached when the glycated hemoglobin level is less than 7% and the blood glucose level within 2 h after a meal is less than 11.1 mmol/L. However, the indicators should be appropriately relaxed for elderly patients and patients prone to hypoglycemia[21]. Regardless of the size of the initial ulcer area, early intensive blood glucose control may improve the prognosis of DFUs in the first 4 wk of treatment[22]. Intensive blood glucose control reduced the risk of amputation in patients with DFUs and contributed to wound healing[23,24]. However, in another systematic analysis, no evidence was obtained that strict control of blood glucose improved ulcer wound healing[25]. Many factors affect ulcer healing, and an increasing number of large samples randomized controlled trials (RCTs) are needed to indicate the effect of intensive blood glucose control on the prognosis of DFUs. According to the specific conditions of patients and blood glucose control objectives, appropriate hypoglycemic programs are formulated to avoid hypoglycemia. Fifty percent of patients with DFUs may have PAD, suggesting that they have atherosclerotic cardiovascular disease[26-28]. According to the latest recommendations of the American Diabetes Association for patients with type 2 diabetes complicated with cardiovascular disease, if blood sugar cannot be controlled by lifestyle changes and metformin, they should start taking a hypoglycemic drug that has been suggested to reduce adverse cardiovascular events and cardiovascular mortality[29], such as a sodium glucose cotransporter 2 inhibitor and a glucagon-like peptide-1 receptor agonist. Compared with the placebo, liraglutide did not increase the risk of DFUs but reduced the risk of DFU-related amputation in patients with type 2 diabetes mellitus and high-risk cardiovascular events[30]. However, the specific mechanism remains unclear. Studies have suggested that liraglutide may promote diabetic wound healing by inhibiting endothelial dysfunction induced by hyperglycemia[31]. Daggligin significantly reduced the level of inflammation and oxidative stress in diabetic animal models, which may contribute to the improvement of endothelial dysfunction and diabetic vascular complications[32]. However, some studies suggested that the amputation risk of patients who use canagliflozin is increased, particularly for patients with DFUs presenting lower limb atherosclerosis, neuropathy and amputation history[33]. However, the OBSERVE-4D study[34] indicated that although cagelin increases the risk of amputation, the risk islower than that reported in previous CANVAS and CANVAS-R trials [35], especially for patients undergoing timely monitoring, who have a lower risk. The results of a randomized controlled trial conducted by Marfella *et al*[36] suggested that the granulation score of wound granulation tissue and the rate of complete wound healing in the Vigliptin group (50 mg/dose, bid) were significantly better than those in the control group, and the incidence of ulcer-related adverse events (such as ulcer wound infection, osteomyelitis, honeycomb tissue inflammation, *etc*.) was also significantly reduced, suggesting that venagliptin may improve the healing rate of DFUs possibly by reducing oxidative stress, changing capillary density, increasing angiogenesis and promoting wound healing. Compared with the control group, the healing rate of foot ulcers in the saxagliptin (5 mg/time, qd) group was higher, and the healing time of ulcers was shorter. The main mechanism was that shagliptin directly and indirectly promoted the epithelial-mesenchymal transformation and reduced scarring to improve diabetic wound healing[37]. Dipeptidyl peptidase IV (DDP-IV) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists reduce inflammatory reactions and antioxidant activity, induce angiogenesis and tissue reconstruction, and may promote DFU healing[38]. These treatments represent new directions with potential effects on DFUs. Systemic insulin therapy improves wound healing of diabetic ulcers by increasing angiogenesis and granulation tissue formation and reducing the duration of the inflammatory phase[39]. Compared with patients with type 2 diabetes using insulin and insulin secretion-promoting drugs, patients with type 2 diabetes using insulin sensitizers have a lower incidence of PAD, suggesting that insulin sensitizers may reduce the incidence of PAD and its subsequent outcomes[40]. Therefore, when choosing hypoglycemic drugs, the appropriate hypoglycemic regimen should be selected according to the basic situation, blood glucose level, wound condition and other comprehensive factors of patients with DFUs.

Management of PAD

Patients with diabetes are prone to PAD. According to a Chinese multicenter study, the proportion of lower-limb arterial disease in patients with diabetes over 50 years old is 19.5%[41]. For every 1% increase in the glycated hemoglobin level, the risk of peripheral vascular disease increases by 25%-28% [42]. For patients with DFUs, the vascular lesions are mainly located in the tibiofibular artery below the knee. The arterial lumen is narrow or even completely occluded, causing lower limb ischemia, hypoxia, infection, ulcer and even gangrene. More than 80% of patients with DFUs have lower limb ischemia[43], which is an important reason for the difficulty in wound healing. Adequate blood perfusion provides a good metabolic demand for the damaged tissue, while an insufficient blood supply may lead to insufficient nutrients available for wound healing and limited delivery of antibiotics, resulting in a decreased healing capacity and increased amputation risk. Therefore, the arterial blood supply of the lower extremities must be reconstructed to improve and restore the blood flow of the extremities, avoid limb ischemia and necrosis, reduce amputation, and improve the quality of life and survival rate of the patients[26,27]. Patients with PAD have a high risk of cardiovascular and cerebrovascular events. Even after revascularization, the incidence of cardiovascular disease is still high[44]. Therefore, patients should also receive active cardiovascular risk management, including smoking cessation, statins, antiplatelet drugs and intensive blood pressure therapy[28,45]. Smoking is a risk factor for atherosclerotic plaques. Severe peripheral atherosclerosis may lead to stenosis and occlusion of the vascular

lumen with the progression of the disease, which may lead to foot tissue ischemia that causes tissue damage and postpones wound healing[46]. Smoking is also a risk factor affecting the degree of DFU lesions[47] and is an effective predictor of death and amputation in patients with DFUs[48]. Smoking cessation is recommended for all patients with PAD. Blood pressure should be controlled within 130/80 mmHg to reduce the risk of cardiovascular and cerebrovascular events[49]. However, another study suggested that the optimal mean systolic blood pressure of patients with PAD was 135–145 mmHg and diastolic blood pressure was 60–90 mmHg. Low blood pressure may increase the risk of cardiovascular events[50]. The use of angiotensin converting enzyme inhibitors (ACEIs) and angiotensin-receptor blockers (ARBs) by patients with PAD not only reduces blood pressure but also reduces major cardiovascular adverse events and mortality[51,52], but it does not reduce major adverse limb events and amputation risk in patients with PAD[52]. Treatments regulating lipid levels target low-density lipoprotein cholesterol. Researchers have recommended a low-density lipoprotein cholesterol (LDL-C) level < 1.4 mmol/L (< 55 mg/dL) or a decrease in the LDL-C level by 50%[53]. In patients with PAD, patients who took statins had an 18% reduction in the long-term risk of adverse prognosis of the lower limbs (such as symptom deterioration, peripheral vascular reconstruction and ischemic amputation) and a 17% reduction in the incidence of cardiovascular events compared with patients who did not take statins, indicating that statins not only reduce the risk of adverse cardiovascular events but also exert a positive effect on the limb prognosis of patients with PAD[54]. Therefore, patients with type 2 diabetes and PAD should be prescribed statins. Medications for improving the circulation of patients with PAD include vasodilator drugs, antiplatelet drugs and anticoagulant drugs. Vasodilator drugs include alprostadil injection, beraprost sodium, cilostazol, salgrel hydrochloride, buflomedil and pentoxifylline, which reduce blood viscosity and change hypercoagulability. Aspirin and clopidogrel are considered antiplatelet drugs. Current practice guidelines recommend the use of aspirin or clopidogrel alone as a method for the secondary prevention of cardiovascular events in patients with PAD[55,56]. Compared with aspirin alone, clopidogrel combined with aspirin significantly reduces all-cause mortality and cardiovascular events, but the risk of severe bleeding is increased[57]. The COMPASS study suggested that the absolute benefit of low-dose rivalsaban (2.5 mg bid) combined with aspirin (100 mg qd) in reducing the risk of cardiovascular events and all-cause mortality in patients with stable atherosclerosis seems to be greater than that of nondiabetic patients[58]. Compared with aspirin alone, low-dose rivalsaban combined with aspirin reduced major cardiovascular events and major limb adverse events. Rivalsaban alone only reduced major limb adverse events but did not significantly reduce major cardiovascular adverse events. However, the latter two schemes increased the risk of bleeding, mainly in the gastrointestinal tract, but the incidence of fatal bleeding or bleeding in key organs did not increase [59]. Routine use of proton pump inhibitors may reduce bleeding from gastroduodenal lesions[60]. Therefore, the combination of low-dose rivalsaban and aspirin provides a new therapeutic direction for patients with diabetes complicated with PAD, but further studies are necessary to determine which subgroups of patients may benefit.

Debridement and management of infection

Patients with diabetes have hypoimmunity, slow ulcer healing and a high infection rate. Infection often occurs when pus flows around the wound and the surrounding tissue is red and swollen. DFUs are usually chronic wounds. The bacteria on the wound surface produce biofilms that inhibit wound healing. Biofilms induce inflammation in the surrounding tissues and adversely affect the removal of bacteria by antibiotics or the host immune system[61]. Therefore, the wound must be thoroughly cleaned. Debridement is the first and foremost measurement of DFU treatment, which involves removing necrotic, inactivated or seriously polluted tissues from the wound surface to convert the wound into an acute wound and facilitate wound healing[62]. Debridement should be performed as soon as possible.

At present, many methods, such as surgical debridement, maggot debridement, high-pressure fluid irrigation and enzymatic treatment, have been developed, but surgical debridement is usually preferred. Amputation is necessary when the ulcer infection worsens or osteomyelitis occurs. For patients with lower limb ischemia, the time of debridement and revascularization must be evaluated. For patients with dry gangrene, the blood supply should be reconstructed first, and then wound debridement should be performed to promote wound healing. However, if wet gangrene or abscess formation is observed in the wound, debridement is preferred. More than half of patients with DFUs had wound infections at the time of the visit. Infection is an important reason for hospitalization of patients with diabetes and an important factor contributing to the nontraumatic amputation of the lower limbs[63].

In the initial stage of superficial DFU infection, gram-positive cocci are mainly detected, among which *Staphylococcus aureus* and *Streptococcus* are the most common organisms[64,65]. If chronic infection, extensive necrosis, wet gangrene, deep infection, long-term repeated use of antibiotics and other conditions exist on the wound surface, a mixed bacterial infection is usually present. Currently, the proportion of gram-negative bacterial infections is increasing, and the proportion of fungal infections is also increasing[66]. Due to the autoimmune status of the body, sanitary conditions, repeated hospitalization, frequent use and abuse of antibiotics, multiple microbial infections, insufficient arterial blood supply of the lower limbs and other reasons, the number of multidrug-resistant bacteria is increasing. The most resistant pathogen is methicillin-resistant *Staphylococcus aureus*[67]. Therefore, accurate identi-

fication of the pathogen causing the bacterial infection is essential for anti-infection treatment of DFUs. Once the infection is confirmed, pathogenic bacteria samples should be collected after the necrotic tissues are removed from the infected wound and before the use of antibacterial drugs. Pathogenic bacterial culture samples shall be obtained from deep tissues as far as possible and sent for culture immediately after the samples are collected. In addition, samples should be collected repeatedly during anti-infection treatment to identify pathogenic bacteria and guide the selection of antibiotics. Tissue biopsy is considered the most useful and standard technology, but it may cause the spread of infection and the loss of adjacent tissue structure of limbs; thus, it is not completely feasible. The collection of swab culture samples is easier, and any type of ulcer can be used. However, the cotton swab culture results usually include colonized bacteria, and the test results are not necessarily reliable[68]. Compared with the culture method, the molecular test method is more sensitive and reliable, with high accuracy and a fast test speed. It represents a powerful method or the identification of microbial colonies infecting chronic wounds and has a bright future in the convenient nursing and treatment of DFUs[69]. Molecular microbiological diagnostic techniques improve the prognosis of patients with chronic wounds[70].

The use of antibiotics should follow the principles of selectivity, timeliness, relatively narrow spectrum, shortest course of treatment, safety, minimal adverse reactions, high cost performance, and step-down. At present, the Infectious Diseases Society of America/International Working Group on the Diabetic Foot (IWGDF) is used to score DFU infection, which is divided into mild (superficial with slight cellulitis), moderate (deeper or more extensive) or severe (with systemic sepsis signs), and the presence of osteomyelitis[71].

The course of antibiotic use is related to the severity of the wound and the presence of bone tissue involvement. The course of treatment ranges from 1 to 12 wk[61]. However, a comprehensive and individualized analysis is necessary to appropriately adjust the course of antibiotics according to the basic diseases of the whole body, nutritional status, liver and kidney functions, blood supply of the lower limbs, and other parameters. Oral antibiotics and intravenous antibiotics maybe selected, but the narrowest-spectrum antibiotic and the shortest course of treatment for pathogenic bacteria should be selected to prevent drug resistance. The IWGDF recommendations[72] for superficial ulcers with localized soft tissue infection (mild) are to start with empirical oral antibiotic treatment against *Staphylococcus aureus* and *Streptococcus aureus* (unless other pathogens should be considered). For deep or extensive infections (moderate or severe infections), a broad-spectrum antibiotic should initially be intravenously administered that mainly targets common gram-positive and gram-negative bacteria, including specific anaerobic bacteria, and the antibiotic program should be adjusted according to the clinical efficacy of empirical therapy, tissue culture and drug sensitivity results. Biofilms are polysaccharide layers formed by a variety of signal transduction mechanisms that delay the healing of DFUs. Therefore, inhibiting the formation of biofilms is a new direction of modern anti-infection treatment research. Studies have shown that acyl homoserine lactones (AHLs) regulate multiple factors during biofilm formation by *Pseudomonas aeruginosa* and play a fundamental role in regulating different genes involved in biofilm formation. Therefore, AHL can be used as a therapeutic target to provide a correct path for drug design targeting multidrug-resistant bacteria[73]. However, in practice, the abuse of antibiotics still frequently leads to the emergence of drug-resistant bacteria. Biological maggot debridement therapy (MDT) provides a new option for the treatment of DFUs.

MDT refers to a natural biological therapy that uses medical maggots to help clean ulcerated wounds by eating the necrotic tissue and bacteria that hinder wound healing[74]; it is used for wounds with unclear boundaries between necrotic tissue and normal tissue, gaps or deep sinuses. It has anti-infection functions, promoting wound growth and debridement. However, because maggots need a moist living environment, ischemic DFUs are not suitable. Currently, the most commonly used organism is the larva of the green silk fly, which is strictly saprophytic and will not cause damage to healthy tissues[75]. Maggots resist infection by draining bacteria from the wound[76,77], absorbing and digesting bacteria in the necrotic tissue of the wound[78], changing the pH of the wound, secreting a variety of bactericidal and antibacterial substances (such as allantoin, urea, phenylacetic acid, calcium bicarbonate, peptides, and bactericides)[79-81], hindering the formation of and degrading bacterial biofilms[82], improving tissue oxygenation at the wound[83], and stimulating the growth of human fibroblasts[84]. Maggots also activate inflammatory cells and other mechanisms to promote wound tissue growth. The necrotic tissue can be cleared through the proteolytic enzymes produced by maggots (such as chymotrypsin, trypsin and collagenase)[85,86] to achieve the debridement effect. Studies have suggested that MDT is effective against a variety of bacterial infections, especially *Staphylococcus aureus*[87], which is resistant to multiple antibiotics. MDT reduces the number of bacteria and decreases the antibiotic treatment time and the hospitalization cost.

In the local treatment of wounds, antiseptics have more extensive antibacterial activity than antibiotics, and they do not induce drug resistance[88]. Antiseptics are commonly used to reduce the bacterial load of wounds and prevent or treat infections[89]. However, the finding that the antiseptic may be toxic to wound-healing cells is a cause for concern, which limits its application. Therefore, the current guidelines recommend the use of clean water or saline to clean the DFUs as the standard of care. In vitro studies of povidone iodine have shown that it penetrates and reduces the formation of biofilms, and it seems to have no negative effect on wound healing[90].

Dressing

After effective debridement of the wound, dressings are applied to keep the base of the wound moist, control the exudation of the wound, avoid normal skin impregnation around the wound, help clean the chronic wound, promote the formation of epithelium and heal the wound. The choice of wound dressings for patients with DFUs should be based on the patient's specific conditions (such as the wound appearance, depth, exudate, infection, compliance and economic status) and the cost and comfort of dressings. Hydrogel dressings, film dressings, foam dressings, hydrocolloid dressings, and alginate dressings are commonly used in clinical practice[91]; see Table 1 for detailed descriptions of various dressings. Hydrogel dressings are widely used in patients with DFUs. These dressings can expand and absorb water and exudate, maintain structural stability, and promote cell proliferation and differentiation and wound healing[92,93]. Some studies have shown that hydrogel dressings shorten the wound healing time by 7.28 d on average and improve the healing rate by 57%[94]. Alginate dressings absorb large amounts of water and can absorb 20 times their own weight. They are the best choice for highly exudative wounds[95]. Synthetic foam dressings can be selected for severe exudative wounds and concave wounds to fill cavities and eliminate potential cavities[96]. In an international, multicenter, double-blind, randomized, controlled, 20-week trial, the noninfectious diabetic neuroischemic foot ulcer had an area greater than 1 cm^2 compared with the control group receiving the same standard of care. After the use of sucrose octasulfate dressing, the number of patients exhibiting wound closure was greater, the wound healing time was shorter, the wound closure rate was improved, and the safety was similar between the two groups[96]. In 2019, the international national guidelines for the prevention and treatment of diabetic foot recommended the use of asucrose octasulfate dressing to promote wound healing of noninfectious neuro-ischemic DFUs that are difficult to heal after standard care[72]. Different dressings have their own advantages. They are selected in the clinic according to the specific conditions of the patient's wound and the characteristics of various dressings (Table 1).

Growth factors and platelet-rich plasma

Growth factors play an important role in wound healing. Vascular growth factors promote the formation of vascular collateral circulation and improve the blood supply of the lower limbs; these factors include fibroblast growth factor and hepatocyte growth factor[97,98]. Platelet-derived growth factor, which is mainly released from platelets, is also released from other cells involved in wound healing, such as endothelial cells, macrophages, keratinocytes and fibroblasts. Platelet-derived growth factor stimulates the secretion of vascular endothelial growth factor and promotes angiogenesis, fibroblast activity, granulation tissue formation and endothelial cell migration. However, a risk of tumorigenesis has been noted, and its application is limited. A systematic analysis was performed to study the effects of 11 different growth factors on DFUs. The local application of growth factors may increase the possibility of a complete cure of foot ulcers in patients with diabetes, but the quality of evidence is low[99]. The safety of using growth factors and the overall reports of their adverse events are very poor, and a comparison of the time points at which various growth factors promote wound healing has not been performed. Further tests are needed to study the effects of growth factors on wound healing. Platelet-rich plasma (PRP) is an autogenous product containing a large amount of platelets, growth factors, fibrin and other substances necessary for wound healing[100]. It is often used to treat relatively sterile wounds after debridement, which potentially improves the proliferation of local granulation tissue in ischemic wounds and promotes wound healing. Compared with standard treatment, topical application of PRP increases the healing potential and promotes complete wound healing without significant adverse events, although the quality of evidence is low[100]. Although growth factors and PRP may promote the healing of DFUs from the perspective of the path-ophysiological mechanism, greater requirements are present on the wound surface. Usually, DFUs are complicated with infections and bacterial biofilms exist, which limits the therapeutic application of growth factors and PRP.

Oxygen therapy

DFUs are often chronic wounds; as a continuous oxygen supply is essential for chronic and difficult wounds[101], oxygen therapy has emerged as a potential treatment. Currently, hyperbaric oxygen and local oxygen therapy are commonly used oxygen therapies. Hyperbaric oxygen therapy is applied *via* a hyperbaric oxygen chamber to reduce inflammatory reactions[102] and induce angiogenesis to promote wound healing, thereby reducing the amputation rate[103]. However, the efficacy of hyperbaric oxygen on DFUs is still controversial. In 1987, *Diabetes Care* published the first cohort study showing that hyperbaric oxygen significantly reduces the amputation rate of patients with DFUs[104]. Follow-up studies also found that when the traditional treatment method for chronic DFUs is not effective, hyperbaric oxygen treatment improves long-term wound healing[105,106]. However, in the study by Margolis, patients with DFUs who were treated with hyperbaric oxygen did not show significantly improved wound healing and amputation rates compared with the control group[107]. Dr. Fedorko *et al* [108] also confirmed that hyperbaric oxygen did not significantly improve the quality of life of patients with DFUs. At the same time, the results of two other RCTs evaluating the use of hyperbaric oxygen in the treatment of DFUs also showed that hyperbaric oxygen therapy did not reduce amputation or

Table 1 Introduction to common clinical dressings[91]

Type of dressing	Character	Scope of application	Advantage	Shortcoming
Film dressing	The polyurethane film is used as a protective layer or a second layer of dressing	Clean and superficial wounds	Good air permeability, isolating bacteria and liquid, transparent film, easy to observe the wound, less immersion, no pain	Strong adhesiveness, non-absorption, easy accumulation of wound exudates, leading to easy growth of bacteria and infections, and impermeability of proteins and drugs
Foam dressing	It is composed of polyurethane or a silicone resin center with a semi-closed outer layer	Burns, chronic wounds, cavity wounds, deep ulcers	Strong water absorption, local humid environment, free from bacteria, easy to use and low cost	Strong adhesiveness, forming an opaque layer, hindering wound observation, unsuitable for dry wounds, and poor stability
Hydrogel dressing	The composition is 70%-90% water and cross-linked insoluble starch polymer; super absorbent resin	Most wound and burn types	Supplement water to maintain a humid environment, high exudation, poor adhesion, easy to remove, accelerate wound healing, reduces pain and inflammation, and low cost	Translucent, semipermeable to gas and water vapor, poor bacterial barrier, sometimes poor mechanical stability, frequent replacement needed, and may cause secondary damage to the wound
Alginate dressing	Alginate is composed of calcium alginate and a calcium–sodium complex, forming a gel on the wound surface to promote Hemostasis	Surgical wounds and burns	Strong water absorption, non-adhesion, high stability, easy to be removed by salt water, and good bacterial barrier	Expensive, smelly, scarce materials, difficult to handle
Hydrocolloid dressing	It is composed of viscous materials, hydrophilic colloids, artificial elastomers, and other components that contact wound exudates to form gels and exert its functions	Chronic ulcers and burns	Strong water absorption, salt water or sterilized water is easy to clean and remove, not easy to adhere, high density, good waterproof performance, and no pain	Slight cytotoxicity, unstable volume, easy leakage of exudate, delayed healing of dextran hydrocolloid, impermeable, unpleasant smell, and obstructing wound observation

promote wound healing in patients with diabetes complicated with chronic DFUs through comprehensive wound care[109,110], but the results of these two studies were highly biased. At present, the number of studies assessing hyperbaric oxygen therapy for DFUs is small, the level of research evidence is low, the efficacy evaluation indicators are uneven, and many subjective factors are present. At the same time, hyperbaric oxygen is time-consuming, expensive, and cost-effective, which restricts the recommendation of hyperbaric oxygen therapy for DFUs. Local oxygen therapy directly delivers oxygen to the wound by pressurization. A multicenter randomized double-blind controlled study showed that standard treatment supplemented with local oxygen therapy increased the possibility of wound healing by more than 4.5 times[111], but more studies are needed to further confirm its efficacy.

Biological scaffold

Biological therapy has been used to promote diabetic wound healing. A study assessing the effect of biological scaffolds (chitosan polyvinyl alcohol and polycaprolactone chitosan polyvinyl alcohol nanofiber blend scaffolds) on the treatment of diabetic rats indicated that the scaffolds had higher biological performance. Compared with the control group, the ulcer area of diabetic rats treated with biological scaffolds was smaller at all time points, and the healing effect was significantly better. At the same time, more obvious granulation tissue was detected in the scaffold-treated wounds[112].

Stem cells/exosomes/cell matrix

As a new technology for the treatment of DFUs, stem cell transplantation may promote neovascularization of the ischemic limb and improve and restore the blood flow of the limb to achieve the goal of treating limb ischemia and the ultimate goal of promoting ulcer healing. Stem cells that have been used in preclinical and clinical research include umbilical cord blood mesenchymal stem cells, umbilical cord mesenchymal stem cells, placental mesenchymal stem cells, adipose mesenchymal stem cells and bone marrow mesenchymal stem cells, among which adipose mesenchymal stem cells are the most widely used cell type. In addition, genetically engineered SCs that overexpress certain cytokines exhibit different characteristics from conventional stem cells *in vivo*, providing a new direction for future clinical applications[113]. The most frequently studied stem cells are mainly found in bone marrow, adipose tissue, cartilage and bone tissue, umbilical cord blood and placenta, of which bone marrow is the most abundant source and these stem cells have a multidirectional differentiation potential, differentiating into osteoblasts, chondroblasts, adipoblasts, muscle cells and nerve cells. Bone marrow mesenchymal stem cells rebuild the local microcirculation[114], improve the blood flow of chronic ischemic limbs[115], provide media and sufficient nutrition for wound repair and remove local metabolites. Pilot research on local transplantation of bone marrow mesenchymal cells for the treatment of patients with vascular disease involving DFUs and lower limbs that failed vascular reconstruction showed that the transplantation of these cells significantly improved the percutaneous oxygen partial

pressure and toe brachial index of the patients, and the limb preservation rate was 81%[116]. Similarly, another study showed that bone marrow mesenchymal stem cell therapy significantly improves the painless walking distance and wound healing of patients with diabetes presenting lower limb vascular occlusion[117]. After 6 mo of treatment with adipose mesenchymal stem cells, approximately 2/3 of the clinical symptoms of lower limb ischemia in patients with diabetes were relieved (including resting pain and walking distance), and angiography showed a significant increase in collateral circulation[118]. A study of nondiabetic patients with lower limb ischemia who could not undergo vascular reconstruction also showed that adipose mesenchymal stem cell transplantation improved their lower limb percutaneous oxygen partial pressure and promoted the healing of local ulcers, proving that this therapy is also applicable to nondiabetic patients with lower limb ischemia[119]. After an intravascular injection of umbilical cord blood mesenchymal stem cells into rats with diabetic skin ulcers, neovascularization in the ulcer area was substantially increased on the third day, new granulation tissue appeared on the seventh day, and stratified squamous epithelial tissue appeared on the fourteenth day. Based on observations on the seventh and fourteenth days, the skin ulcer area was significantly reduced. The mechanism was that the injected umbilical cord blood mesenchymal stem cells promoted the secretion of keratin 19 by epithelial keratinocytes, participating in the formation of extracellular matrix [120]. Bone marrow mesenchymal stem cells not only improve vascular disease in the lower limbs of patients with diabetes but also promote the healing of ulcers[117,121,122]. Studies have shown that adipose mesenchymal stem cells also contribute to the healing of skin ulcers in diabetic mice[123,124]. At present, the mechanism of action of stem cells remains unclear. Generally, mesenchymal stem cells directly from new blood vessels through endocrine secretion from vascular endothelial cells and smooth muscle cells that participate in angiogenesis through paracrine vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, angiopoietin-2, angiopoietin-1 and other angiogenic factors[125]; improve the local microcirculation; increase the blood supply of the distal foot; and promote the healing of DFUs, but the mechanism still requires further study.

With the research and disclosure of the mechanism of action of stem cells, the development of stem cells or other cell derivatives with clearer mechanisms of action for DFU treatment has become a research hotspot. The application of these derivatives in DFU treatment shows efficacy and characteristics similar to those of stem cells. Among these derivatives, exosomes are the most popular. Exosomes are spherical or cup-shaped vesicles surrounded by double-layer membranes that are secreted by various cells. They exist in saliva, blood, milk, semen, blood and other tissue fluids. Exosomes carry a variety of signaling molecules and bioactive substances and participate in the occurrence and development of systemic immunity, intercellular communication, cell proliferation, cell migration, cell differentiation and metabolic diseases[126]. Previous studies have confirmed that mesenchymal stem cells exert therapeutic effects on DFU. They release exosomes through paracrine signaling, and exosomes, important mediators of intercellular communication, participate in the appellate cell process, which is one of the mechanisms by which mesenchymal stem cells exert their therapeutic functions. Exosomes secreted by mesenchymal stem cells are membrane vesicles with a diameter of 30-150 nm and a density of 1.10-1.18 g/mL[127]; they carry nucleic acid molecules such as miRNAs, cytosolic proteins and mRNAs and bind to receptors to mediate intracellular signal transduction and change cell functions. Exosomes secreted by adipose mesenchymal stem cells promote the proliferation of vascular endothelial stem cells, angiogenesis, wound granulation tissue formation, and growth factor expression and reduce the levels of inflammation- and oxidative stress-related proteins in a high-glucose environment[128]. Exosomes secreted by umbilical cord blood mesenchymal stem cells induce wound angiogenesis[129]. In a study that treated chronic wound skin of diabetic rats with exosomes secreted by PRP, exosomes effectively induced the proliferation and migration of fibroblasts and endothelial cells and improved the angiogenesis and re-epithelialization of chronic wounds[130]. Mesenchymal stem cell exosomes derived from menstrual blood also promote neovascularization and increase the amount of neovascularization in the skin of diabetic mice[131]. Exosomes are characterized by a simple structure, lack of replicability, lack of genetic material in the stem cell nucleus, shorter action time, smaller particle size for easy diffusion in vivo, and other properties. At the same time, *in vivo* studies have confirmed that they have similar biological activity to stem cells intreating DFUs. Therefore, stem cell derivatives with higher safety and simpler mechanisms of action will have better development prospects. However, exosomes are also associated with various problems, such as a high preparation cost, hampering large-scale production. Methods to produce uniform and reliable exocrine therapeutic drugs is an important research topic for the clinical application of exosomes in the future.

Due to the inconvenience caused by the characteristics of stem cells in preparations for the external use of stem cells, an increasing amount of research is devoted to the development of excipients that provide support for stem cells, such as collagen scaffolds and cell gels, to prolong the maintenance of the efficacy of preparations for the external use of stem cells. The extracellular matrix (ECM) is a noncellular three-dimensional polymer network composed of collagen, elastin, proteoglycan/glycosaminoglycan, laminin, fibronectin and other glycoproteins[132]. It provides extracellular scaffolds for cells and interacts with cells. The ECM directly or indirectly affects the shape, metabolism, migration, proliferation and apoptosis of cells. Studies have confirmed that it is closely related to immunity, inflammation, angiogenesis, wound healing and malignant transformation[133]; therefore, maintaining ECM homeostasis is very important for DFU healing. Hyaluronic acid is an important component of the

skin ECM. It affects many processes, such as cell migration, proliferation, inflammatory reactions and angiogenesis, in the proliferation stage of wound healing and plays an important role in wound healing and tissue repair[134]. However, the hyaluronic acid content is reduced in DFU skin, resulting in delayed wound healing[135]. Collagen promotes myofibroblast differentiation and fibrosis to maintain the ECM structure and promote healing[136], but collagen deposition in DFU skin reduces the skin thickness and integrity[137]. In individuals with diabetes, the production of matrix metalloproteinases in the ECM increases, and the ratio of matrix metalloproteinases/tissue inhibitors of metalloproteinases increases, aggravating the inflammatory response and leading to an imbalance in ECM homeostasis [138]. In DFU wound tissues, the secretion of matrix metalloproteinases is increased, and the levels of matrix metalloprotease-hydrolyzed ECM fragments are increased. Studies have confirmed that the occurrence of inflammation *in vivo* is significantly related to the presence of a large number of ECM fragments and their receptors[139]. At the same time, the production of ECM fragments in the inflammatory process also activates immune cells, leading to the continuous occurrence of inflammatory reactions[140]. The interaction between the abnormally expressed ECM and the inflammatory response causes a high level of inflammation to persist in DFU wounds for a long time and makes wound healing difficult. In view of the complexity and safety of the stem cell therapy mechanism and the special disease characteristics of DFUs, the development of stem cell preparations for the external treatment of DFUs, such as those combined with ECMs caffolds, is another effective technical approach showing considerable application prospects and will certainly play an important role in future clinical applications.

Negative pressure wound therapy

Negative pressure wound therapy (NPWT) includes two modes: vacuum-assisted closure (VAC) and vacuum sealing drainage (VSD). The pipeline used by VAC has poor hydrophilicity and a high supporting force. The pipeline is placed on the surface of the dressing to form a device similar to a suction cup. Wound healing is promoted by adjusting the negative pressure level and selecting the intermittent mode[141]. The drainage tube adopted by VSD has high plasticity, good hydrophilicity and contains side holes. It covers the dressings and wound surface with a fully closed and translucent polyurethane film to form a closed space. The necrotic tissues and secretions on the wound surface are drained by negative pressure to promote wound cleaning; it is mainly used for drainage of deep wounds and body cavities. VAC focuses on the treatment of wounds on the body surface and exerts a good effect on treating DFUs, limb soft tissue lacerations, lower limb venous ulcers, deep pressure ulcers, and other wounds[142-144].

NPWT is widely used to treat DFUs as an acute and chronic wound treatment technology. Armstrong *et al*[142] suggested that, compared with standard treatment, VAC accelerated wound healing, improved wound healing ratio and reduced the re-amputation rate when treating complicated diabetic foot wounds. In the meta-analysis by Liu *et al*[145], compared with conventional dressing changes, VAC reduced the area and depth of DFU to a greater extent, improved the complete healing rate of ulcer, shortened the healing time of ulcer, reduced the amputation rate of patients, and improved cost-effectiveness.

The mechanism of NPWT is as follows: (1) Keep the wound moist and stabilize the wound environment pain[146]; and (2) inhibit bacterial growth. Weed *et al*[147] indicated that after treatment with negative pressure drainage technology, the number of bacteria in the wound, particularly gram-negative bacteria, was significantly reduced. Additional components of the mechanism include: (1) Improving wound blood perfusion and promoting wound healing[148]; (2) promoting cell proliferation, angiogenesis and wound tissue repair[149]; and (3) regulating the signaling pathway to modulate cytokine expression[150].

Before using NPWT, the necrotic tissue and dead bone on the wound surface should be completely removed. NPWT also has contraindications, such as deep wound infection, severe ischemia, eschar or necrosis, active bleeding, coagulation dysfunction, exposure of blood vessels or nerves or tendons or ligaments, untreated osteomyelitis, wet gangrene, and malignant tumors. At the same time, the use of NPWT may lead to tube plugging, poor drainage, residual dressings, wound maceration, residual dressings, and other complications.

Based on the limitations of NPWT, negative pressure wound therapy with installation (NEWTi) emerged at a historic moment. It combines negative pressure therapy with liquid perfusion technology to accelerate the cooperative use of wound water and promote the dissolution and clearance of deep necrotic tissues by intermittently or continuously perfusing solutions to closed wounds. The destruction of biofilms and autolytic debridement are the main factors contributing to the superiority of NPWTi to NPWT. However, a uniform standard for the selection of irrigation solution, irrigation time, irrigation speed, and irrigation frequency is unavailable when NPWTi is used to treat DFUs.

Offloading

At present, the basic principle for the prevention and treatment of neurogenic DFUs is to redistribute the increased local pressure on the foot[151,152], reduce the plantar pressure and shear force, and promote wound healing. Therefore, the selection of a suitable decompression device according to the actual situation of the patient is very important to prevent foot ulcers[153]. Offloading is divided into

nonsurgical offloading and surgical offloading. Nonsurgical offloading provides external decompression through the use of individually customized or prefabricated devices. The efficacy is determined by whether the patient can continue to wear the decompression device. Currently, the most commonly used pressure reducing devices are the total contact cast (TCC) and detachable cast. TCC is the most effective decompression technique for the treatment of neurogenic DFUs[154] and is even the gold standard for foot restraint and treatment of DFUs[14]. Studies have shown that TCC can reduce the pressure at the ulcer by 84%-92%[155], and it is effective for most nonischemic and noninfectious diabetic plantar ulcers, with an ulcer healing rate of 69.6%-73.9%[156,157]. A TCC can reduce local inflammation, help reduce or delay edema during wound healing, accelerate ulcer healing, and possibly protect the foot from infection because it is not easy to disassemble, which increases the patient's compliance with use and may be an important reason for its benefit[158,159]. However, as TCCs are not easy to disassemble and patients may easily fall while wearing them, their use limits patients' daily activities. Moreover, inappropriately fitting braces may cause skin irritation, even skin ulceration and infection, and muscle weakness may occur after long-term use. At the same time, TCC use requires the cooperation of experienced doctors, technicians and patients, and consequently, the application of TCCs is limited. The detachable plaster branch has the advantages of easy disassembly, easy observation of wounds, convenient local treatment of wounds, and it can be used for the treatment of infected wounds and superficial ulcers. However, it also has the disadvantages of poor patient compliance and the reduction of the local decompression effect due to irregular wearing. Therefore, patients must be educated while repeatedly emphasizing the benefits of consistently wearing the device to achieve the therapeutic effect[160]. For patients who do not accept gypsum braces, felt-like foam pads and appropriate therapeutic shoes can also be used for decompression treatment[72]. Studies have confirmed that therapeutic shoes reduce plantar pressure and prevent ulcer recurrence[161]. Crutches, walking aids and wheelchairs may also be used for decompression, but some devices increase the pressure on the healthy foot during use, which will increase the incidence of new ulcers on the healthy side[155]. At the same time, the use of these devices is limited due to the lack of upper limb strength and perseverance to use these devices independently[162].

Surgical decompression treatment serves to redistribute the pressure or change the position of the pressure points through surgery, with the purpose of permanently changing the internal pressure point. When a patient does not exhibit complete local decompression after using the optimized shoes and tools and ulcers occur or the patient cannot decompress after ulcer healing and after using the optimized shoes and tools, then he or she can be decompressed during amputation. Surgical decompression usually includes Achilles tendon extension, metatarsal head resection, arthroplasty, and toe flexor tendon resection[163]. Patients with diabetes are prone to shortening of the calf gastrocnemius muscle, which will lead to a continuous increase in the plantar pressure of the forefoot. The extension of the Achilles tendon may reduce the plantar pressure of the forefoot[164]. Studies have confirmed that Achilles tendon lengthening promotes wound healing and reduces ulcer recurrence in patients with neuropathic plantar ulcers and horseshoe foot[165]. Diabetic neuropathy leads to a high pressure load on the plantar skin above the metatarsal head. Removing these biomechanical factors may reduce pressure and facilitate wound healing. For nerve plantar ulcers with difficult healing, early removal of the metatarsal head may be the key to promoting wound ulcer healing[166]. Compared with traditional conservative treatment, metatarsal head resection has a higher healing rate and lower infection rate and ulcer recurrence rate[167]. At the same time, metatarsal head resection promotes the healing of plantar ulcers, which is not related to sex, age, body mass index, height, weight, diabetes duration or the duration of preoperative ulcers[168]. Arthroplasty is an effective procedure for the treatment of recurrent or complex neurological DFUs. Using routine treatment and decompression, metatarsal finger arthroplasty results in a faster healing rate and a lower recurrence rate than the standard treatment [169]. The flexor pollicis longus and flexor digitorum longus can be used to decompress the toe and make the toe tip more flexible[170]; a systematic review confirmed that this operation exerts a good therapeutic effect on closing wounds and newly formed ulcers[171].

Low-level laser therapy

Low-level laser therapy (LLLT) uses low-energy light to stimulate the wound surface and produce a series of pathophysiological reactions mainly through photobiological regulation. It does not directly induce photothermal injury of the wound tissue and does not damage the normal tissue cells at the wound surface[172]. In the study by Kaviani *et al*[173], 8 patients in the LLLT group achieved complete healing after 20 wk, while only 3 patients in the control group achieved complete healing. Although the difference was not statistically significant, the average time of complete healing in patients receiving LLLT (11 wk) was less than that in the control group (14 wk), suggesting that LLLT might accelerate the healing process of chronic DFUs and shorten the time of complete healing, but the sample size of this experiment was small. Another analysis of the efficacy of LLLT in the process of chronic wound tissue repair of DFUs showed that the tissue repair index in the LLLT treatment group increased significantly, mainly because LLLT shortened the inflammatory period, promoted angiogenesis and the production of extracellular matrix components, and accelerated the healing process[173,174]. Percival *et al*[175] proposed that LLLT promotes wound healing by inhibiting the microbial membrane of chronic wounds, especially cocci and some gram-negative bacteria. Other studies have shown that LLLT promotes

wound healing by improving the blood flow and autonomic nervous system regulation of DFUs[176]. A systematic review and meta-analysis of the efficacy of low-dose laser treatment of DFUs[177] found that the ulcer area in the LLLT treatment group was significantly reduced by 30.90% compared with the control group. Compared with the control group, the ulcer area in the treatment group decreased by 4.2 cm^2. The probability of complete healing of DFUs was 4.65 times higher than that of the control group, indicating that LLLT may accelerate wound healing and reduce the area of DFUs. However, the review did not provide the best laser treatment parameters. However, in another review, LLLT was shown to be safe and effective in treating DFUs. The laser parameters were 632.8-685 nm, 50 mW/cm^2, and 3–6 J/cm^2; the irradiation time was 30–80 s, three times a week, and the duration of one month was beneficial for the prognosis of DFU wounds in patients[178]. Because the pathophysiological mechanism of DFUs is complex and the prognosis of the ulcer surface is different due to the diverse ulcer surfaces and different laser parameters, more rigorous, high-quality and large-sample RCTs are needed to determine the best treatment parameters for different types of ulcers.

PREVENTION

The incidence of DFUs is high, and the amputation rate is high; moreover, ulcer healing is slow, and the treatment effect is relatively poor. Therefore, the prevention of DFUs is particularly important. However, people currently focus on treatment after the occurrence of DFUs. Researchers mainly focus on medical treatment rather than prevention. Cesare Miranda[179] suggested that comprehensive management should be implemented to prevent DFUs and provided a flow chart for the prevention of DFUs, including DFU education, blood glucose control, management of PAD, identification of risky feet, regular inspection of susceptible feet, long-term wearing of appropriate shoes, and treatment of ulcer risk factors. DFU education can reduce the incidence of DFUs and amputations. It encourages patients to conduct foot self-examinations, identify risk factors, provide appropriate self-care and treat feet with any signs of pre-ulceration[153]. However, the smooth performance of this examination is usually affected by the decrease in vision and limited movement of patients. Through regular foot screening and follow-up of patients with diabetes, the incidence of DFUs and the amputation rate have significantly decreased, but only 20%-30% of patients in China undergo regular foot screening[180]. Studies have shown that the use of diabetic foot treatment shoes and insoles may reduce the ulcer recurrence rate by 30%-50%, but the ulcer recurrence rate is still as high as 30%[181]. In the study by Frykberg *et al*[182], a new type of remote wireless intelligent temperature monitoring foot pad system was provided for patients with previous DFUs, which is a wireless temperature foot pad that can be used in daily life and senses changes in and asymmetry of foot temperature. The research results show that the intelligent detection system accurately predicts the experimental patients with recurrent DFUs. However, this experiment has its own limitations, including its nonintervention design, small sample size, short experimental time, lack of evaluation of other factors and costs that may affect the occurrence of DFUs, and artificial bias. The results thus require further confirmation. Nevertheless, the results of this study are still very meaningful, suggesting that more intelligent devices can be further developed for the prevention and treatment of DFUs and can thereby reduce the familial and social burdens related to patients with DFUs.

CONCLUSION

DFUs are one of the serious complications of diabetes. Many risk factors lead to the occurrence of the disease, and the amputation rate is high. Once diabetes is diagnosed, we should perform more work on the management of diabetes, including screening for high-risk factors for DFUs, such as neuropathy and arteriopathy. The high incidence of DFUs may be related to the lack of DFU risk education programs, the insufficient attention of patients, the low rate of foot examination, and the poor knowledge of medical personnel. Boulton *et al*[183] found that less than 20% of patients with diabetes received a foot examination provided by medical and health professionals. Therefore, foot care education should be provided to all patients with diabetes and the risk of DFU should be evaluated at a minimum of annually[184].

With the development of artificial intelligence, intelligent detection instruments and evaluation tools (such as intelligent insoles) can be applied to the prevention and treatment of DFUs. The management of DFUs requires multidisciplinary cooperation, mainly including endocrinologists, vascular surgeons, orthopedic doctors, wound specialists, shoe technicians, rehabilitation physicians, psychological consultants and specialized nurses. The correct evaluation and comprehensive management of DFUs by multidisciplinary teams are essential to protect the function and quality of life of patients. Optimizing diabetes management is still the most important step to prevent diabetes-related complications[185, 186]. The effect of intensive treatment on the prognosis of DFUs requires further study. In patients with diabetes complicated with PAD, sodium glucose co-transporter 2 inhibitors or GLP-1 receptor agonists are recommended[145,187]. Both DDP-IV inhibitors and GLP-1 receptor agonists promote DFU healing

[187]. When patients with DFUs choose hypoglycemic drugs, they should not only consider the hypoglycemic effect but also consider whether cardiovascular risk factors are present and whether these drugs can promote ulcer healing. Statins, antiplatelet agents, ACEIs and ARBs are effective in the secondary prevention of cardiovascular events in patients with PAD. For patients with PAD complicated with diabetes, the combination of low-dose rivarsaban and aspirin reduces major limb adverse events, including amputation[60], but the risk of bleeding must be monitored. Offloading relieves plantar pressure and shear force to promote wound healing. The value of decompression shoes lies in preventing ulcers, not in using them during the treatment of active ulcers[188]. Surgical offloading is mainly employed to treat specific foot ulcers, usually when other nonsurgical offloading interventions fail. Internal offloading and external offloading are used together to promote wound healing. Necrotic tissue and the microbial membrane are removed, and chronic wounds are transformed into acute wounds. A systematic review reviewed the effect of surgical debridement on DFU healing. The results indicated that the higher the application frequency of surgical debridement, the better the results[189]. However, excessive debridement is not conducive to ulcer healing. An appropriate debridement frequency and debridement method should be selected for different DFUs. The combined use of antibiotics, wound dressings and NPWT may accelerate wound healing. However, the efficacy of oxygen therapy must be confirmed in more high-quality studies. Additionally, the specific parameters of LLLT treatment for different DFUs also require strict, large-sample RCTs researches to provide data. Biological scaffolds, stem cells, exosomes, cell matrix, growth factors and PRP represent new approaches for the treatment of DFUs. The preliminary data seemed positive and revealed a potential effect, but the specific mechanisms of action of these therapies are not clear, and further clinical research may provide better suggestions. In summary, multidisciplinary combination treatment should be adopted in the treatment of DFUs.

The current situation is that the screening rate and follow-up rate of DFUs are low, the incidence rate and the amputation rate are high, and many treatment methods are available, but the effect is not satisfactory. However, with the development of the information age, people's understanding of diabetes and DFU has gradually improved, and various new technologies have been continuously developed, which provides opportunities for the management of DFUs. In the future, comprehensive prevention and treatment management of DFUs are needed to avoid the occurrence of DFUs, effectively shorten the healing time of DFUs, improve the clinical cure rate, reduce the amputation rate, improve the standard of living of patients with DFUs, and reduce the social burden. This task may be a complex, huge and meaningful project.

FOOTNOTES

Author contributions: Yang L and Rong GC contributed equally to this work; Yang L, Rong GC and Wu QN contributed to writing the manuscript and participated in helpful discussions; Wu QN is the guarantor of this work.

Supported by Chongqing Science and Technology Bureau and Health Commission of Chinese Medicine Technology Innovation and Application Development Project, No. 2020ZY013540; General Project of Graduate Education and Teaching Reform of Chongqing University, No. cquyjg20329; Science and Health Joint Project of Dazu District Science and Technology Bureau, No. DZKJ,2022CCC1001.

Conflict-of-interest statement: The authors have no conflicts of interest and agreed to publish the study.

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S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1035-1048

DOI: 10.4239/wjd.v13.i12.1035

ISSN 1948-9358 (online)

REVIEW

Keeping an eye on the diabetic foot: The connection between diabetic eye disease and wound healing in the lower extremity

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Morya AK, India; Wu QN, China

Received: August 28, 2022
Peer-review started: August 28, 2022
First decision: September 26, 2022
Revised: October 27, 2022
Accepted: November 18, 2022
Article in press: November 18, 2022
Published online: December 15, 2022

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Abstract

Diabetic eye disease is strongly associated with the development of diabetic foot ulcers (DFUs). DFUs are a common and significant complication of diabetes mellitus (DM) that arise from a combination of micro- and macrovascular compromise. Hyperglycemia and associated metabolic dysfunction in DM lead to impaired wound healing, immune dysregulation, peripheral vascular disease, and diabetic neuropathy that predisposes the lower extremities to repetitive injury and progressive tissue damage that may ultimately necessitate amputation. Diabetic retinopathy (DR) is caused by cumulative damage to the retinal microvasculature from hyperglycemia and other diabetes-associated factors. The severity of DR is closely associated with the development of DFUs and the need for lower extremity revascularization procedures and/or amputation. Like the lower extremity, the eye may also suffer end-organ damage from macrovascular compromise in the form of cranial neuropathies that impair its motility, cause optic neuropathy, or result in partial or complete blindness. Additionally, poor perfusion of the eye can cause ischemic retinopathy leading to the development of proliferative diabetic retinopathy or neovascular glaucoma, both serious, vision-threatening conditions. Finally, diabetic corneal ulcers and DFUs share many aspects of impaired wound healing resulting from neurovascular, sensory, and immunologic compromise. Notably, alterations in serum biomarkers, such as hemoglobin A1c, ceruloplasmin, creatinine, low-density lipoprotein, and high-density lipoprotein, are associated with both DR and DFUs. Monitoring these parameters can aid in prognosticating long-term outcomes and shed light on shared pathogenic mechanisms that lead to end-organ damage. The frequent co-occurrence of diabetic eye and foot problems mandate that patients affected by either condition undergo reciprocal comprehensive eye and foot evaluations in addition to optimizing diabetes management.

Key Words: Foot ulcer; Diabetic; Wound healing; Diabetes complications; Amputation; Diabetic retinopathy; Corneal ulcer

Core Tip: This review explores the epidemiological and pathophysiological interconnections between diabetic foot and eye disease, especially the shared mechanisms that impact wound healing. Since diabetic foot and eye problems are often concurrent, it is imperative that patients affected by one or the other condition promptly undergo reciprocal examinations to reduce the risk of further complications. The best outcomes for patients with diabetic foot and eye disease are achieved by a team-based strategy that incorporates regular examinations, often performed by specialists, provides preventative health education, and delivers effective long-term management of the underlying diabetes and its associated metabolic consequences.

Citation: Ramsey DJ, Kwan JT, Sharma A. Keeping an eye on the diabetic foot: The connection between diabetic eye disease and wound healing in the lower extremity. *World J Diabetes* 2022; 13(12): 1035-1048
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1035.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1035

INTRODUCTION

An estimated 131 million people worldwide have lower extremity complications related to diabetes mellitus (DM), such as diabetic foot ulcers (DFUs), peripheral vascular disease (PVD), neuropathy, and amputations[1,2]. Similarly, an estimated 103 million people worldwide have diabetic eye disease, including nearly one million people aged 50 and older who are blind from diabetic retinopathy (DR)[3,4]. The frequent co-occurrence of diabetic eye and foot problems makes it imperative for patients affected by either condition to promptly undergo reciprocal examinations to reduce the risk of further complications (Figure 1). It is essential that individuals who have DM and the clinicians who care for them understand the likelihood of this association. With the worldwide prevalence of DM increasing because of changes in diet and lifestyle, aging of the population, and the ability of individuals to live longer with the disease, the need for well-informed clinicians has never been greater[3].

The connection between diabetic eye and foot problems is related, in part, to shared risk factors. In particular, the duration of DM and level of glycemic control as reflected by hemoglobin A1c (HbA1c) level strongly govern both the rate of onset and severity of diabetic foot disease[5,6] and DR[7]. Molecular biomarkers, particularly ceruloplasmin, have been demonstrated to be elevated in people with DM[8]. Other risk factors, such as age, male gender, race and ethnicity, smoking, insulin use, type of diabetes, and individual comorbid factors such as hypertension, elevated low-density lipoprotein (LDL), decreased high-density lipoprotein (HDL), coronary artery disease, cerebral vascular disease, PVD, neuropathy, and nephropathy, have been assessed, but not all studies agree on which of these risk factors affect the incidence or progression of these diabetic complications[5]. Some of this variation may possibly be ascribed to differences in DM care, improvements in treatment over time, and other less well-defined differences between individual populations studied. This paper reviews the shared pathogenic mechanisms underlying these conditions and the importance of comprehensive diabetes care to reduce morbidity and prevent disability.

DIABETES-ASSOCIATED LOWER EXTREMITY COMPLICATIONS AND THEIR OCULAR PARALLELS

DFUs

Individuals with DM are at a significantly increased risk of developing DFUs. DFUs are full-thickness wounds that penetrate the dermis (the deep vascular and collagenous inner layer of the skin) and are located below the ankle in patients with both type 1 and type 2 DM (Figure 2)[1,2]. DFUs arise from a combination of micro- and macrovascular compromise related to hyperglycemia and associated metabolic dysfunction that causes impaired growth and wound healing, immune dysregulation, and PVD[9]. In addition, the loss of protective sensation and proprioception caused by diabetic neuropathy and vision loss from diabetic eye disease predisposes patients to repetitive lower extremity trauma, with DFUs a common complication, especially among older adults[10,11]. Risk factors for DFUs include age,

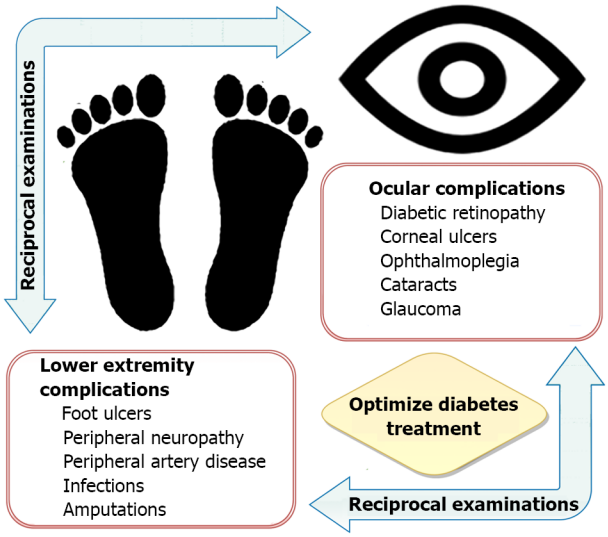

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Figure 1 Upon identification of one or more of problems involving the lower extremity or the eye, reciprocal examinations are recommended to reduce the risk of further complications. Preventing diabetic foot and eye problems is best achieved through regular examinations, diabetes education, and optimal management of underlying diabetes mellitus and its associated metabolic consequences.

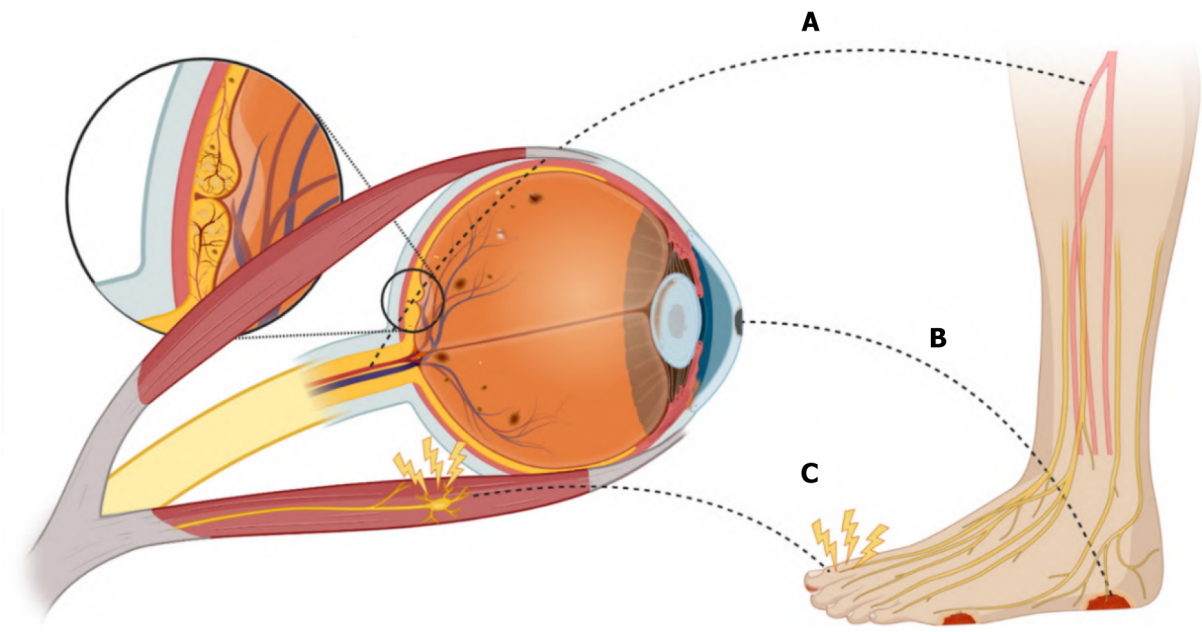

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Figure 2 The connection between diabetic eye disease and diabetic foot and wound healing. A: Diabetic micro- and macrovascular complications; B: Diabetic ulcers; C: Diabetic neuropathy. Each of these diabetic complications has multifactorial etiologies. Figure 2 was made in ©BioRender-biorender.com.

deformity or prior ulceration, repetitive trauma, sensory and autonomic neuropathy, peripheral arterial disease (PAD), and infection[12]. Up to one third of patients with DM will be affected by a DFU in their lifetime[13]. DFUs are associated with a 10- to 20-fold increased risk of amputation[1] and have a one-year mortality rate as high as 5%[14]. As many as 20% of DFUs remain unhealed after one year of treatment, with unhealed ulcers posing a risk for infections, gangrene, amputation, and even death[15, 16].

There is a strong association between the development of DFUs and DR (Figure 2)[5]. Depending on the population studied, most individuals affected by DFUs also have DR[6,17-19], and those with DR are two to four times more likely to have DFUs or more serious forms of diabetic foot disease (Table 1)[20-22]. Even more concerning is the strong association between DFUs and proliferative diabetic retinopathy (PDR), with 31% to 55% of individuals having this more severe stage of DR (see below)[23]. Furthermore, patients with nonproliferative diabetic retinopathy (NPDR) who develop comorbid non-

Table 1 Studies examining association of diabetic foot disease and diabetic retinopathy

Ref.	Year	Type of study	Sample size (DFU; no DFU)	Source of population	Main findings
Jayaprakash *et al*[17]	2009	Prospective Case Study	94	India	73.4% prevalence of DR in patients with DFUs
Hwang *et al*[22]	2017	Retrospective Cohort	100	South Korea	90% prevalence of DR in patients with type 2 DM and DFUs; 55% had PDR
Karam *et al*[18]	2018	Cross-sectional	182	India	67.6% prevalence of DR in patients diabetic foot disease (including neuropathy, deformation, DFUs, or amputation)
Zafar *et al*[19]	2019	Cross-sectional	530 (225; 305)	Pakistan	96% of patients with DFUs had DR
Sellman *et al*[23]	2020	Case Control	270 (90; 180)	Sweden	31% prevalence of PDR in patients with DFUs
Banik *et al*[21]	2020	Cross-sectional	680 (8; 672)	Bangladesh	65.9% prevalence of DR in patients with DFUs
Ye *et al*[20]	2014	Retrospective Cohort	829 (61; 768)	China	OR 2.026 for DFUs in patients with DR
Al-Rubeaan *et al*[6]	2015	Retrospective Cohort	62681 (2071; 60610)	Saudi Arabia	OR 4.45 for diabetic foot disease (including DFUs, gangrene, and amputation) in patients with DR
Harris Nwanyanwu *et al*[24]	2013	Retrospective Cohort	4617	United States	1.54 HR for those with comorbid non-healing DFUs to progress from NPDR to PDR in three to five years

DR: Diabetic retinopathy; NPDR: Nonproliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; DFU: Diabetic foot ulcer; OR: Odds ratio; HR: Hazard ratios.

healing DFUs have a greater than 50% increased risk of progressing to PDR relative to those without this condition[24]. Finally, diabetic keratopathy is an important ocular parallel of DFUs. It is a disruption of normal corneal wound healing and loss of protective mechanisms of corneal sensation and aqueous tear production. These aberrations create an ideal environment for persistent corneal epithelial defects, microbial infection, and ulceration. Around half of patients with DM are affected by this condition[25]. The pathophysiology accounting for diabetic structural and functional alterations in the cornea is discussed in depth below.

Microvascular complications

Microvascular dysfunction in the lower extremity contributes to impaired function and impedes wound healing, which promotes the development of DFUs. Damage to endothelial cells from chronic hyperglycemia, oxidative stress-induced injury, generation of advanced glycation end-products, increased polyol flux regulated by aldose reductase, activation of protein kinase C (PKC), and other pro-inflammatory processes from immune dysregulation cumulatively disrupt normal blood flow and affect vascular permeability[26]. In its most severe form, this compromise of the microvasculature leads to ischemia and a relative hypoxic state in the involved tissue. As a result, there is increased expression of hypoxia-inducible factor-1 (HIF-1) leading to the production of vascular endothelial growth factor (VEGF), a protein principally responsible for restorative angiogenesis[27]. However, in DM there are disturbances in cytokine growth factor expression and locally decreased concentrations of VEGF, which render the lower extremity vulnerable to poor wound healing[9]. Failure of the microvasculature also contributes to peripheral neuropathy and local immune dysfunction, including impaired cellular response, cytokine expression, and vascular tone[28].

In the eye, these same pathways driven by hyperglycemia and other diabetes-associated factors lead to progressive damage to the retinal microvasculature and cause the development of DR[29]. DR develops in roughly one quarter of patients with DM, with the prevalence being highest in Africa (36%) and lowest in South and Central America (13%)[3,30]. Initially, the disease manifests as clinically detectable changes in the retinal vasculature, including the development of microaneurysms and loss of capillaries which are the hallmarks of early NPDR[31]. As the disease progresses, the production of VEGF and other diabetes-associated factors promotes further dysfunction, vascular leakage, and bleeding (dot-blot hemorrhages)[32]. At this stage, visual acuity is increasingly likely to be affected and is often further limited by swelling in the center of the retina, known as diabetic macular edema (DME)[33]. The development of neovascularization on the optic nerve or at locations in the peripheral retina signifies the progression to PDR. This is the most vision-threatening complications of the disease also primarily driven by the abnormal expression of VEGF[29,30,34]. These fragile new vessels, which grow into the vitreous cavity and along the inner retinal surface, often bleed, causing vitreous hemorrhages, traction, or retinal detachment and thereby impair vision[3,32]. Finally, excessive expression of VEGF may also affect the anterior segment of the eye by causing neovascularization on the iris and ciliary body. When the growth of this fibrovascular tissue extends to the anterior chamber angle it may block

outflow of aqueous humor through the trabecular meshwork causing eye pressure to rise to levels capable of damaging the optic nerve in a disease process known as neovascular glaucoma[4,32]. When left unaddressed, irreversible blindness results.

Clinical examination supplemented by diagnostic color fundus photography and fluorescein angiography are the mainstays for staging DR; however, emerging modalities including ultrawide-angle imaging and optical coherence tomography and angiography increasingly allow clinicians to directly and noninvasively visualize the diseased retina and its microvasculature[31]. Advances in therapeutic modalities, such as intraocular injection of agents that target VEGF, steroids that target inflammation, and panretinal laser photocoagulation, have improved clinical outcomes for patients with DR[33-36]. However, effective long-term management is largely dependent upon regular follow-up care. Patients who fail to return for care are more likely to suffer vision loss[37,38].

The intraocular administration of agents that target VEGF are now the most common treatments for DR and DME[29,36]. Thankfully these agents are very unlikely to negatively impact wound healing in the lower extremity, especially at the doses employed to treat eye disease[39]. Similarly, intraocular corticosteroids, such as dexamethasone, and intravitreal steroid implants utilized to treat DME have been found to have no detectable influence on HbA1c or renal function[40,41]. However, when larger doses are administered as subconjunctival or peribulbar injections, some patients can experience elevations of blood glucose, similar to that observed with oral and intravenous administration of corticosteroids[42]. Finally, topical steroid drops have only very rarely been associated with endocrinological side effects in case reports[43].

Wound healing

Wound healing in the lower extremity requires coordinated cellular responses that cause an organized release of growth factors and cytokines. Under normal conditions, when an injury occurs, multiple cell types, including macrophages, fibroblasts, and epithelial cells, release VEGF and other cytokines in response to local ischemia caused by the wound[44]. However, in patients with DM, disturbances in cytokine and growth factor expression, including fibroblast growth factor, insulin-like growth factor, platelet derived growth factor, and VEGF, among others, lead to a condition that subsequently permits prolonged hypoxia[9,45]. Additionally, keratinocytes and fibroblasts in DFUs have demonstrated attenuated cellular migration, proliferation, and protein synthesis, resulting in impaired re-epithelialization which further exacerbates the oxygen-restricted wound[46]. Moreover, hyperglycemic states reduce the stability and function of HIF-1, which further impairs the wound healing response as a downstream consequence of sustained hypoxia[9]. Increased free radical damage is also a known causative factor in impaired wound healing in patients with DM. Inappropriately elevated concentrations of reactive oxygen species (ROS) and impaired antioxidant enzyme activity can cause nerve damage and directly contribute to the progression of peripheral neuropathy[47].

Individuals with DM also have abnormal wound healing pathways in the eye. Notably, corneal thinning is thought to be the earliest detectable pathological manifestation of DM in the eye[48]. Diabetic keratopathy leads to persistent corneal epithelial defects and neurotrophic corneal ulcers that respond poorly to treatments applied in the hyperglycemic environment[49]. Abnormalities in corneal cell morphology, varied number and disorganization of epithelial cell layers, impaired cellular migration, reduced endothelial cell number, and accumulation of acellular debris all contribute to poor wound healing[50]. Sectorial thinning, bullae, and persistent corneal epithelial defects from diabetic keratopathy often lead to corneal ulcers, scarring, and reactive neovascularization, which cause decreased visual acuity or permanent vision loss[50,51]. Although the cornea itself is avascular and ischemia does not play a significant role in diabetic keratopathy, wound healing in the cornea, like in the lower extremity, requires highly structured cellular processes which are impacted by hyperglycemia. These involve proliferation and migration of epithelial cells, fibroblasts, and the expression of numerous growth factors, including transforming growth factor beta, epidermal growth factor, insulin-like growth factor, and platelet derived growth factor[51]. Finally, diabetes-associated hyperglycemia may also impair vision by accelerating the progression of diabetic cataract and impact the health of the lens epithelium[52].

Most treatments for diabetic foot and eye problems are applied locally, but some treatments to aid the lower extremity may have theoretical consequences on the eye, and vice versa. Several adjuvant therapies have been found to reduce DFU healing times and amputation rates, including non-surgical debridement agents, topical dressings and agents, negative pressure wound therapy, oxygen therapies, acellular bioproducts, and human growth factors[53]. Oxygen is required for almost every step of the wound healing, affecting cell proliferation, collagen synthesis, and re-epithelialization, as well as immunologic defense against bacteria and other pathogens[54]. Oxygen may be delivered in the form of local, hyperbaric, or supplemental inspired oxygen therapy. Hyperbaric oxygen therapy has proven to be particularly useful in managing chronic, non-healing DFUs, especially in the relatively ischemic diabetic foot, albeit at a high financial cost[55]. Patients have been observed to have increased tissue concentrations of VEGF after completing hyperbaric therapy sessions; this has been attributed to the sharp decline of relative oxygen concentration once a session is completed[56]. As previously mentioned, the presence of VEGF is the primary driver of DR, so there is a theoretical risk that systemic or local oxygen therapy could exacerbate this condition. However, empiric evidence does not suggest

that oxygen therapy is harmful to the diabetic eye[23]. It has even been reported that patients with concurrent DR have benefitted from the administration of hyperbaric oxygen therapy through supranormal levels of oxygen delivered to the retina[57]. Nevertheless, it remains essential that the status of DR is assessed and regularly monitored in any patient undergoing oxygen therapy for DFUs.

Many growth factors that have been identified as integral to wound healing are also potential therapeutic targets. In the diabetic foot, among the most promising are hydrogels which contain recombinant PDGF, approved by the United States Food and Drug Administration for topical administration having demonstrated improved rates of DFU healing in randomized clinical trials[58]. In the diabetic cornea, recombinant human nerve growth factor (NGF), epidermal growth factor, and metalloprotease inhibitors have demonstrated some success in trials for the treatment of diabetic keratopathy [59]. Of note, the opioid antagonist naltrexone has been demonstrated to improve wound healing, corneal surface sensitivity, and tear secretion in diabetic animal models[60,61]. The future will also likely include gene- and cell-based therapies to accelerate wound healing, including in DFUs and diabetic cornea[62,63].

Diabetic neuropathy

Approximately half of adults with DM will be affected by peripheral neuropathy in their lifetime[64]. Peripheral neuropathy typically begins with diminution or loss of protective sensation. In addition, loss of proprioception contributes to injuries and falls[11]. Moreover, autonomic dysregulation in the foot may contribute to impaired cutaneous blood flow, sweating dysfunction, and loss of vascular tone that compromise integument integrity and wound healing[65]. Lower extremity deformities may also occur, such as hammer toes or claw toes, which are associated with loss of function[11]. Finally, delays in the identification of accidental and iatrogenic injuries because of reduced sensation may cause patients to fail to seek care in a timely fashion and increase the risk for infections[64].

As mentioned above, chronic hyperglycemia from DM causes microangiopathic changes. In the case of diabetic neuropathy, hyperglycemia may affect the endoneurial microvasculature by directly reducing perfusion and impairing nerve function[66]. Many of the same cellular and biochemical mechanisms linked to chronic hyperglycemia injure the peripheral nerves, including increased glycolytic processes producing oxidative stress, generation of advanced glycation end-products, increased polyol flux regulated by aldose reductase, PKC activation, and other pro-inflammatory processes from immune dysregulation[67]. Damage to mitochondria also plays an important role in the pathogenesis of diabetic neuropathy and contributes to nerve dysfunction, cell death, and loss of neurotrophic support provided by neurotrophin-3 and NGF[68]. Patients who develop PAD also have more severe diabetic neuropathy (see below)[64].

The cornea is the most densely innervated tissue of the human body and is 100 times more sensitive than skin[69], but this declines with age[70] and is further reduced by DM[71]. The loss of protective sensation of the diabetic cornea impacts various homeostatic functions, such as blinking, aqueous tear production, and the release of growth factors[51,52]. As a result, the incidence of dry eye disease and the need for artificial tears is increased among patients with DM, particularly among those with worse diabetes-related outcomes[72]. A recent meta-analysis estimated that DM conferred 30% increased odds for dry eye syndrome[58]. Dry eye disease and DR are also associated with each other[73]. These changes result in neurotrophic keratopathy marked by persistent epithelial defects and chronic erosions that may develop into corneal ulcers, corneal scarring, and neovascularization, all of which contribute to visual dysfunction[74] and predispose patients to infectious keratitis[75]. The recent application of *in vivo* confocal microscopy has allowed for visualization of diabetes-associated structural changes in the nerves of the corneal epithelium, including nerve thickening[76] and decreased nerve length and density[77]. Anterior segment optical coherence tomography is another emerging diagnostic modality used to evaluate and manage diabetic keratopathy by enabling the direct visualization of the cornea structure and nerves[50,71].

Diabetes-associated hyperglycemia has also been shown to cause direct injury to the neuronal retina, leading to thinning of the nerve fiber layer from the loss of ganglion cells and the death of other retinal neurons, including photoreceptors[78]. This may lead to decreased visual function, impaired contrast sensitivity, and diminished night vision[29,32]. Finally, the eye may also be suddenly and directly affected by diabetic cranial neuropathies, manifesting as double vision from ophthalmoplegia, which is the paralysis of the muscles that move the eye (see below).

Current recommendations for the management of painful diabetic neuropathy include gabapentinoids, serotonin and norepinephrine reuptake inhibitors (SNRIs), and tricyclic antidepressants (TCAs)[79]. Gabapentin is a well-tolerated medication from an ophthalmic standpoint; its most common adverse effect is a reversible nystagmus[80]. SNRIs, such as venlafaxine, have been associated with acute angle closure glaucoma in some case reports[81], along with increased cataract development[82]. Finally, TCAs are associated with blurred vision in up to one third of patients, likely due to the anticholinergic action of these drugs[83]. These side effects further emphasize the importance of communication and collaboration with ophthalmologists when treating diabetes-associated complications of the lower extremity.

Macrovascular complications

Common macrovascular complications of DM that affect the lower extremity include PAD and chronic venous insufficiency (CVI), which may lead to lower extremity amputation (Figure 1)[84]. DM induces and accelerates the development of atherosclerosis *via* multiple mechanisms that include metabolic derangements, smooth muscle dysfunction, oxidative stress, potentiated platelet function, increased coagulability, and chronic inflammation[85]. The availability of the potent vasodilator nitrous oxide, which is produced in the endothelium and is a primary mediator in local vascular endothelial tone, is reduced in hyperglycemic states; DM also promotes the production of endothelin-1, which indirectly increases vasoconstriction and vascular smooth muscle hypertrophy[86]. The result may be an overt occlusion, sometimes acutely when a thrombus forms, or when increasingly stenotic vessels result in reduced perfusion[86]. Patients with co-existing severe PAD are also more likely to have CVI[84], which contributes to poor wound healing by increasing hydrostatic pressure in the lower extremity, thereby promoting wound exudation[87].

While not directly a macrovascular complication, it is important to recognize that DR is strongly associated with lower-extremity PAD. Patients with DR have an approximately two-fold increase in the need for lower-limb revascularization and a five-fold increase in lower-limb amputation[88]. Patients with PAD benefit from additional medical management and risk factor modification for atherosclerotic disease. In addition to optimizing diabetes control, this includes counseling about smoking cessation, antiplatelet and statin therapies, as well as blood pressure control[89]. Exercise also plays a fundamental role in the treatment of PAD, leading to reductions in pain and improvement in functional capacity[89]. The clinical benefit of newer medications on amputation prevention remains uncertain.

In the eye, DM-associated macrovascular disease can manifest as an ocular ischemic syndrome (OIS), a rare, but vision-threatening condition associated with severe carotid artery occlusive disease that leads to ocular hypoperfusion[90]. Like PAD, atherosclerosis affecting the vessels supplying the eye is the main cause of the disease, and most patients with OIS have a diagnosis of DM[91]. Patients typically report dull eye or periorbital pain associated with gradual vision loss as the retina experiences progressive ischemia. Consequently, VEGF levels rise, which may cause neovascular glaucoma in the anterior segment and reduce the final visual potential; neovascularization can also develop in the retina, but it is less prominent than in DR because of reduced retinal perfusion[92]. OIS entails an overall poor visual prognosis, which means that the ophthalmologist's diagnosis is crucial for the systemic health of those patients because OIS may be the presenting sign of impending serious cerebrovascular and ischemic heart disease. Finally, DM can sometimes cause an ischemic optic neuropathy, which is a direct infarct of the optic nerve[52].

Another condition involving a main function of the eye where macrovascular disease in DM manifests is ophthalmoplegia. Ophthalmoplegia is the paralysis of one or more of the extraocular muscles (EOM). It can arise from traumatic, autoimmune, infectious, and vascular etiologies. Usually involving the third (oculomotor), fourth (trochlear), or sixth (abducens) cranial nerves, double vision is the characteristic symptom of ophthalmoplegia[93]. The vascular supply for the EOMs comes from branches of the ophthalmic artery, which is itself a branch of the internal carotid artery. Additionally, the cranial nerves responsible for the EOMs themselves have a complex vascular supply. Focal cranial nerve ischemia due to atherosclerosis within the microvasculature is thought to contribute to the development of ophthalmoplegia in patients with DM[94].

PREVENTION AND MANAGEMENT

Preventing diabetic foot and eye problems is best achieved through regular examinations, diabetes education, and optimal management of underlying DM and its associated metabolic consequences. Tight control of blood glucose, as reflected by HbA1c level, is the most important element for prevention of these two interrelated diabetes-associated complications, closely followed by optimization of blood pressure and lipid levels[1,7,37,38]. This is accomplished through a combination of regulation of diet, lifestyle modification, body mass reduction, and medications, such as insulin and/or oral antidiabetic therapies, as appropriate. Monitoring alterations in serum biomarkers, such as HbA1c, ceruloplasmin, creatinine, uric acid, LDL, and HDL, is also important because these biomarkers are associated with both the onset and severity of DR and DFUs[8,20].

The standard practices in DFU management include cleansing, surgical debridement, application of clean dressings to maintain a moist environment and control exudates, wound off-loading, vascular optimization (including revascularization procedures), treatment and prevention of infection, and glycemic control[88,95]. Proper instruction is also required to prevent accidental or iatrogenic injuries which can result from ordinary hygiene and grooming of the feet and lower extremity[29]. Infection prevention is best achieved through protective footwear, proper hygiene, and offloading interventions [11]. Similarly, preventing complications from diabetic keratopathy focuses on limiting repetitive trauma, neurosensory deformities, exposure, and infections. Injuries may be caused by eye droppers, abnormal eye lashes, cosmetic applicators, fingers, facial towels, and bedding[96]. Infection can occur from overgrowth of the ocular flora or opportunistic infection enhanced by hyperglycemia, or it can

take the form of chronic and recurrent herpes simplex and zoster[97].

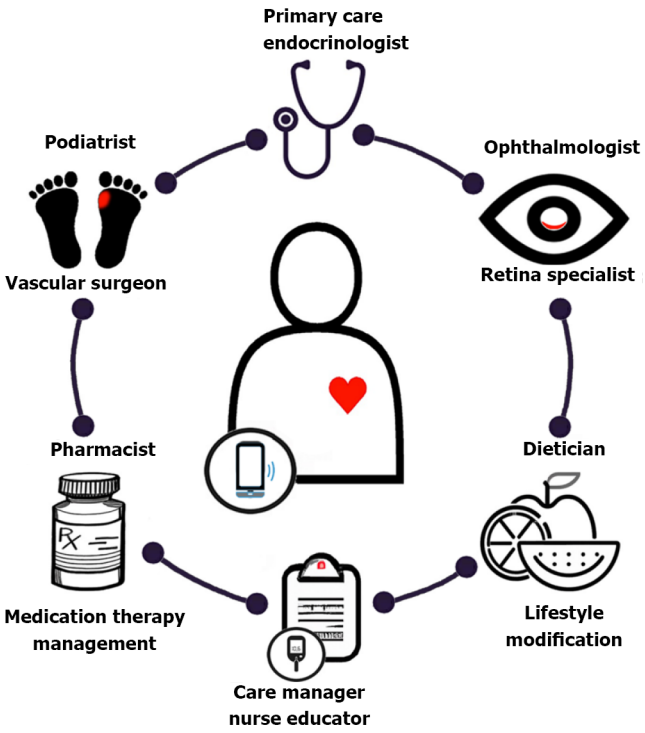
Many parallels exist between management of ulcers in the cornea and those in the lower extremity. Both are treated with clean dressings, antimicrobial ointments, and salves. Wound infections may be polymicrobial, but the bacterial species most associated with DFUs include gram-positive species, *e.g.*, Staphylococcus aureus and Streptococcus species, but gram-negative infections with Pseudomonas aeruginosa and Enterobacteriaceae species also occur and are notably more common in ischemic or deep wounds[98]. Infection of corneal ulcers involve many of these same organisms, including Staphylococcus, Pseudomonas aeruginosa, and Streptococcus pneumoniae[70,99]. Special dressing and vacuum-assisted wound closure have been used with good result in the management of DFUs[100]. Non-healing diabetic corneal ulcers are often treated in conjunction with bandage contact lenses, which can lengthen the time antibiotic treatments are in contact with the ocular surface and serve as a reservoir for pharmacologically active compounds to aid wound healing[69]. In contrast to DFU management, patching should generally be avoided in patients with DM and corneal disease because of an increased risk of infection[101]. Amniotic membrane grafts have been studied for their potential of facilitating epithelial migration and healing of corneal ulcers and in very severe cases, corneal transplantation may be necessary[102].

Emerging research has placed an emphasis on developing therapeutic options that offer additional ways of preventing diabetic complications, treating them at earlier stages, or in more effective ways. As discussed earlier, inflammation has been implicated in the pathogenesis of diabetes-associated complications. Cytokines, such as interferon-γ, are being investigated as potential therapeutic targets in attenuating inflammatory cascades given that many of these cytokines contribute to altered vascular permeability and angiogenesis[103]. Given the high metabolic rate of the retina in conjunction with the metabolic stress induced by chronic hyperglycemia, reducing free radical stress may be an effective strategy[104]. Polyphenols, such as epigallocatechin-3-gallate found in green tea, are known for their antioxidant and anti-inflammatory properties and in diabetic animal models, have been shown to attenuate ROS concentrations in the retina[105]. Other polyphenol compounds, carotenoids, thiols, and vitamin supplementation are being investigated to address the several pathways involved in ROS generation and inflammation[104].

Finally, a multidisciplinary care team is essential to care optimally for the diabetic foot and eye, preserving function and quality of life for those with DM (Figure 3). Primary care providers and endocrinologists play a crucial role in coordinating care, including providing a formal assessment of the degree of diabetic control, screening for symptoms related to diabetic complications affecting other organ systems such as diabetic nephropathy, prescribing DM treatment, and involving specialists who manage diabetic complications such as foot or eye problems[1]. A diabetes care team should also include pharmacists who provide medication therapy management, dieticians, psychologists, diabetes care managers, and nurse educators. By working together, a coordinated care team can effectively reduce the healthcare burden associated with DM and its complications through prevention, screening, and management. In the future, the integration of smartphone technology and telehealth may not only streamline care coordination, but also allow for remote diagnosis and long-term monitoring of disease [106].

CONCLUSION

The identification of any ophthalmic or lower extremity complication in a patient with type 1 or type 2 DM should immediately prompt a review of DM management and coordination of diabetes care, including referral for reciprocal comprehensive foot or eye evaluations in patients with either complication[1,37,38]. Although diabetic foot disease is slightly more common among patients with type 1 DM and those who use insulin[6], optimizing diabetes management remains the most important step in preventing diabetes-associated complications no matter what the type of DM[37,38]. While many patients may report symptoms related to diabetic foot disease or observe vision loss in the setting of diabetic eye problems, many others may be asymptomatic or have such mild signs and symptoms that they are easily overlooked, dismissed, or fail to receive clinical attention unless specifically assessed[64,107]. Primary care providers and endocrinologists should perform regular diabetic foot examinations because they provide insight into the presence and degree of PVD, neuropathy, skin breakdown, and other pre-ulcerative changes. Providers must also screen for signs and symptoms of eye disease, in part because their identification may help triage the urgency of any necessary referrals[37,38]. Because diabetic eye and foot diseases so commonly occur in conjunction, it is essential that clinicians take the necessary steps to reduce the impact of these diseases through regular screening, prompt referral to specialists, and providing a coordinated, team-based approach to management.



DOI: 10.4239/wjd.v13.i12.1035

Figure 3 Diabetic care team process. Primary care and endocrinology physicians are central to comprehensive diabetes mellitus (DM) evaluation including assessment of the level of glycemic control, prescription of medications, determining level of treatment adherence, and identification of gaps in care and risk of complications. The frequent concurrence of diabetic eye and foot problems mandate that patients affected by either condition should undergo reciprocal comprehensive eye and foot evaluations, in addition to optimizing diabetic control. Specialists are often required to manage diabetic foot problems, including referral to podiatry, lower extremity wound care specialists, or vascular surgery, each engaging treatment algorithms according to their expertise. Eye care is typically provided by ophthalmologists or optometrists, but often requires the expertise of a retinal specialist capable of providing the medical and surgical management of diabetic eye disease. Pharmacists provide medication therapy management and are an important sources of diabetes education. Dietitians, lifestyle coaches, and psychologists offer counseling that works toward improving or maintaining glycemic targets through nutrition, achieving weight management and physical activity goals, and implementing behavior changes. Diabetic care managers and nurse educators help individuals with DM establish long-term commitments. They provide instruction on foot and skin care; the use of medications, including the administration of insulin; the monitoring of blood glucose levels; and maintenance of proper diet and exercise. They develop an overall management strategy aimed at reducing risk factors linked to diabetes-associated complications. The integration of smartphone technology and telehealth may streamline the care coordination and communication between the patient and each component of the diabetic care team [106]. Figure 3 was made in ©BioRender-biorender.com. The authors generated parts of the digital images used in Figure 3 by using the Generative Pre-trained Transformer 3 (GPT-3) autoregressive language model that employs deep learning to generate digital images from natural language descriptions (DALL·E, OpenAI, San Francisco, CA, labs.openai.com). The authors reviewed, edited, and revised these images and take ultimate responsibility for the content included in this publication.

ACKNOWLEDGEMENTS

The authors thank Dr. Andrew Popelka Jr., Dr. Shiyoung Roh, Dr. Sarkis Soukiasian, Dr. Adam Romeiser III, Dr. Angela Jellison, Rebecca Rick Longo, as well as Carol Spencer, Lahey Hospital Librarian, for research support. D.J. Ramsey is supported by the Harry N. Lee Family Chair in Innovation at the Lahey Hospital and Medical Center, Beth Israel Lahey Health. Study was performed as part of regular employment duties at the Lahey Hospital and Medical Center, Beth Israel Lahey Health. No additional funding was provided.

FOOTNOTES

Author contributions: Ramsey DJ, Kwan JT, and Sharma A contributed equally to this work; All authors have read and approve the final manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1049-1065

DOI: 10.4239/wjd.v13.i12.1049

ISSN 1948-9358 (online)

REVIEW

Diabetic foot ulcers: Classification, risk factors and management

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Gluvic Z, Serbia; Latiri IO, Tunisia

Received: August 27, 2022
Peer-review started: August 27, 2022
First decision: October 5, 2022
Revised: October 18, 2022
Accepted: November 18, 2022
Article in press: November 18, 2022
Published online: December 15, 2022

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Abstract

Diabetic foot ulceration is a devastating complication of diabetes that is associated with infection, amputation, and death, and is affecting increasing numbers of patients with diabetes mellitus. The pathogenesis of foot ulcers is complex, and different factors play major roles in different stages. The refractory nature of foot ulcer is reflected in that even after healing there is still a high recurrence rate and amputation rate, which means that management and nursing plans need to be considered carefully. The importance of establishment of measures for prevention and management of DFU has been emphasized. Therefore, a validated and appropriate DFU classification matching the progression is necessary for clinical diagnosis and management. In the first part of this review, we list several commonly used classification systems and describe their application conditions, scope, strengths, and limitations; in the second part, we briefly introduce the common risk factors for DFU, such as neuropathy, peripheral artery disease, foot deformities, diabetes complications, and obesity. Focusing on the relationship between the risk factors and DFU progression may facilitate prevention and timely management; in the last part, we emphasize the importance of preventive education, characterize several of the most frequently used management approaches, including glycemic control, exercise, offloading, and infection control, and call for taking into account and weighing the quality of life during the formulation of treatment plans. Multidisciplinary intervention and management of diabetic foot ulcers (DFUs) based on the effective and systematic combination of these three components will contribute to the prevention and treatment of DFUs, and improve their prognosis.

Key Words: Diabetes; Diabetes foot ulceration; Classification; Diabetes complications; Clinical management; Lower limb complications

Core Tip: Diabetic foot ulcers (DFUs) are a common complication of diabetes. The high recurrence and amputation rates associated with DFUs reflect an urgent need to improve care and treatment methods, highlighting the importance of a comprehensive investigation of the important components of clinical diagnosis and treatment. This article reviews the classification and risk factors of DFUs and summarizes the common clinical management approaches.

Citation: Wang X, Yuan CX, Xu B, Yu Z. Diabetic foot ulcers: Classification, risk factors and management. *World J Diabetes* 2022; 13(12): 1049-1065
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1049.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1049

INTRODUCTION

The prevalence of diabetes mellitus (DM) is rapidly spreading at an alarming rate worldwide[1]. DM is known to damage multiple organs, including the heart, kidney, eye, and nerves, leading to complications such as heart attack, stroke, blindness, kidney failure, and lower limb amputation. Diabetic foot ulcer (DFU) is a frequent complication that occurs in approximately 6.3% of patients with DM globally [2]. The high incidence of DFU and the associated mortality and morbidity are the most common reasons for hospitalization of diabetes patients. Early in the course of DM, patients experience serious foot sensitivity symptoms such as pain and tingling, while later stages of the disease course are characterized by negative symptoms such as numbness and weakness of the toes. With the progression of the disease, patients usually show mixed pain sensitivity and dullness, along with decreased limb sensation and motor function, which lead to imbalance and unsteadiness and increase the likelihood of falls[3,4]. In addition, because of the increasing morbidity, DFU is a leading cause of non-traumatic amputation and is associated with an increased risk of death[5].

The high incidence and intractability of DFU extract a substantial cost in terms of reduced productivity and increased healthcare-related expenses. Appropriate and prompt treatment of DFU requires a multifaceted approach, including timely and correct diagnosis and classification, multiple assessments of risk factors, and appropriate choice of management, all of which should be based on the patient's actual condition. This primer will present the current knowledge of the potential pathogenesis of DFU, discuss the clinical classification of DFU, highlight the corresponding approaches to diagnosis and common management techniques, and close with a call that more attention and feasible interventions are required for DFU management.

DEFINITION AND CLASSIFICATION OF DFU

Definition

The practical guidelines formulated by the International Working Group on the Diabetic Foot (IWGDF) defined DFU as a set of symptoms secondary to current or previous diabetes, including skin chapping, ulceration, infection, or destruction of foot tissue, which partly reflects the fuzzy and imprecise nature of this concept[6,7]. DFU is a complicated and multifactorial clinical problem that affects many patients with diabetes, who experience ulceration and infection, invariably with neuropathy and/or peripheral artery disease (PAD), that disrupt the foot epidermis and dermis, breach the skin envelope, expose sterile structures, and finally form full-thickness lesions[8]. In the Western world, more than 60% of non-traumatic amputations involve DFU, which leads to an increase in hospitalization rate and mortality[9] and causes reduced quality of life (QoL). Moreover, treatments based on amputation impose a heavy burden on the economic and health resources of patients with diabetes[10].

Classification

The multiple factors associated with the development of DFU, such as the complex process and complications of diabetes, may all lead to various degrees of neurological abnormalities and vascular damage (known as neuropathy and PAD)[11]. Once the ulcer is formed, the factors affecting healing may be more complex, and different factors may dominate at different stages over time. Thus, these related factors play different roles depending on the severity of disease and duration of recovery, necessitating different diagnoses and treatments for seemingly the same symptoms and causing differences in the curative effect[12]. In these circumstances, the classification and scoring criteria for describing lesions of DFU should be formatted in a manner that is clinically recognized and widely used, which will allow characterization of DFU on the basis of differences and facilitate suggestions for treatment or care programs.

Considering the different audiences and objectives of the classification and scoring systems, no universally accepted system has been published to date. Various systems are used to describe and assess the severity of DFU, and three types of key factors contributing to the scoring system have been proposed, namely, patient-related, limb-related, and ulcer-related factors, which reflect end-stage renal failure, PAD, and loss of protective sensation, along with classification of the wound grade[13]. Most systems set scoring criteria based on the size and characteristics of the wound, such as size, depth, ischemia, and infection, allowing characterization of the lesion, while risk factors such as neuropathy and peripheral arterial occlusive disease are incorporated when clinical interventions or preventive guidance are required[14,15]. In this section, we will introduce several major systems and summarize their characteristics and applications.

The Meggitt-Wagner system: This system, which was described by Meggitt in 1976 and disseminated by Wagner in 1979, was once the most widely used system[16-18]. It is a six-grade classification system mainly covering the depth of the ulcer and the degree of tissue necrosis[19] (Table 1). This system, which is essentially wound-based, is intuitive and simple to use, but since it does not consider clinical parameters such as peripheral neuropathy and PAD, it cannot distinguish between infection and ischemic lesions, which is also related to its recognized imprecision and limitations[20].

The University of Texas classification system: The classification system proposed by the University of Texas (UT) takes some common clinical signals and symptoms of DFU into consideration by using a 4 × 4 matrix assessing ulcer depth horizontally and infection and ischemia status vertically[15,21] (Table 2). Since it aims to divide patients into four categories depending on whether they are infected or ischemic on the premise of distinguishing the depth of ulcer, the UT system is more helpful to predict amputation than the Meggitt-Wagner system, which simply classifies the ulcer condition[21,22].

The size (area, depth), sepsis, arteriopathy, denervation system: The size (area, depth), sepsis, arteriopathy, and denervation [S(AD)SAD] system was proposed in 1999 and is mainly designed for clinical audits[23]. The system was first verified in 2004, and in order to further refine the classification of ulcers for prospective research, some criteria missing from the UT system were included subsequently[24]. This system contains five elements that are scored in grades 0-3 according to severity, namely, size (area, depth), infection (sepsis), ischemia (arteriopathy), and neuropathy (denervation), and uses acronyms to facilitate memorization and feature generalization[24] (Table 3). The advantage of this system lies in its ability to allow specific recording of ulcers without requiring professional testing technology and equipment, facilitating its usage in clinics. However, because of the multiple descriptions of characteristics and irregular details of ulcers, the system is difficult to remember for operators, which may be the reason why the S(AD)SAD system is considered to be more suitable for audits while the UT system is used for clinical description and communication[25,26].

The site, ischemia, neuropathy, bacterial infection, area, depth system: A simplified and refined form of the S(AD)SAD system, the site, ischemia, neuropathy, bacterial infection, area, depth (SINBAD) system, was proposed to reduce the difficulties in clinical use caused by the inclusion of more complicating criteria while retaining the descriptions of ulcer characteristics to the maximal extent possible[12,27]. The SINBAD system still contains five elements (area, depth, infection, ischemia, and neuropathy), and grades each element as either 0 or 1 point to create an evaluation system with scores of 0-6 for description of increasing severity[27] (Table 4). The modified system is simple but sufficiently robust and allows collection of the necessary information without specialist equipment, except for routine clinical examinations[13]. It has been proven to have moderate inter-observer and excellent intra-observer reproducibility and may help accurately describe the progress of ulcers, including healing and the need for amputation, which was confirmed by the fact that IWGDF recommends the SINBAD system[28].

The Wound, Ischemia, and foot Infection system: Because of the rising prevalence of neuroischemic ulcers, the dichotomy for ischemia in the existing systems lacks effective severity grading and cannot meet clinical requirements. In 2014, the Wound, Ischemia, and foot Infection (WIfI) system was proposed by the Society for Vascular Surgery Lower Extremity Guidelines Committee, and it covered the three most important risk factors that may cause amputation of lower limbs: WIfI[29]. The three factors are assigned scores from 0 to 3, of which the wound is graded on the basis of size, depth, severity, and anticipated difficulty in achieving wound healing; ischemia is rated on the basis of ABI gradation; and foot infection is rated on the basis of the scope and depth of the wound[29] (Table 5). Clinical studies have suggested that this system primarily offers value in predicting major amputation [30]. In patients with DFU and vascular disease, the WIfI system is recommended to evaluate perfusion and vascular function and help rapidly implement revascularization and/or drainage[31]. Since the evaluation of foot perfusion indices requires specialist measurements, assessments using this system require expertise in vascular intervention, indicating that it is not ideal for use in primary and/or community care[13].

Table 1 Wagner classification system

Grade	Ulcer depth
0	Pre-ulcerative area without open lesion
1	Superficial ulcer (partial/full thickness)
2	Ulcer creep to tendon, capsule, bone
3	Stage 2 with abscess, osteomyelitis, or joint sepsis
4	Localized gangrene
5	Global foot gangrene

Table 2 University of Texas classification system[21]

	Grade 0	Grade 1	Grade 2	Grade 3
	Pre- or post-ulcerative site	Superficial wound not involving tendon, capsule, or bone	Wound penetrating to tendon or capsule	Ulcer penetrating to bone of joint
Lesions without infection or ischemia				
Infected/non-ischemic lesions				
Ischemic noninfected lesions				
Ischemic infected lesions				

Table 3 Size (area, depth), sepsis, arteriopathy, denervation system

Grade	Size				
	Area	Depth	Sepsis	Arteriopathy	Denervation
0	Skin intact	Skin intact	None	Pedal pulses present	Pin pricks intact
1	< 1 cm^2	Superficial (skin and subcutaneous tissue)	Surface	Pedal pulses reduced or one missing	Pin pricks reduced
2	1-3 cm^2	Tendon, periosteum, joint capsule	Cellulitis	Absence of both pedal pulses	Pin pricks absent
3	> 3 cm^2	Bone or joint space	Osteomyelitis	Gangrene	Charcot

RISK FACTORS FOR DFU

DFU is caused by multiple interacting risk factors, of which the most common major identified factors include diabetic neuropathy (DPN), PAD, and foot deformities. These factors can be further divided into different degrees according to the severity[32-36]. In this section, the main risk factors are listed and introduced.

Neuropathy

The neuropathy induced by diabetes is a symmetric polyneuropathy that affects the sensory, motor, and autonomic components of the peripheral nerves to varying degrees[37]. Epidemiological data shows that neuropathy is responsible for 16%-66% of the cases of diabetic foot syndrome[38], and patients with neuropathy are prone to show relapse after healing, eventually leading to lower limb amputation[39]. DPN results in the loss of protective sensation, usually starting in a symmetrical and sock-like manner. Small and unmyelinated nerve fibers responsible for conducting afferent sensory perception, like C-type fibers, are the first to be damaged, resulting in tissue damage due to poor perception of trauma and/or mechanical stress. Thus, the relatively minor damage will continue to accumulate and result in a progressively worsening wound with difficulty in healing[33].

Motor neuropathy causes atrophy of foot muscles by denervation of specific muscle groups, which directly affect the function of the foot. Since the small muscles of the foot, like the extensor digitorum brevis and lumbrical and interosseous muscles, are paralyzed gradually, the anatomy of the foot arch changes, and the metatarsophalangeal joints (MTPJs) become hyperextended or over-contracted[40,41]. The joints remain movable in the initial stage, but with aggravation of the symptoms, the

Table 4 Site, ischemia, neuropathy, bacterial infection, area, depth system[13]

Category	Definition	Score
Site	Forefoot	0
	Midfoot and hindfoot	1
Ischemia	Pedal blood flow intact: At least one palpable pulse	0
	Clinical evidence of reduced pedal flow	1
Neuropathy	Protective sensation intact	0
	Protective sensation lost	1
Bacterial infection	None	0
	Present	1
Area	Ulcer < 1 cm^2	0
	Ulcer ≥ 1 cm^2	1
Depth	Ulcer confined to skin and subcutaneous tissue	0
	Ulcer reaching muscle, tendon or deeper	1
Total possible score		6

Table 5 Wound, Ischemia, and foot Infection system

Grade	Wound	Ischemia			Foot infection system
	Clinical features	ABI (mmHg)	ASP (mmHg)	Toe pressure, $TcPO_2$ (mmHg)	Clinical manifestations
0	No ulcer no gangrene	≥ 0.80	> 100	≥ 60	No symptoms or signs of infection. Infection present, as defined by the presence of at least two of the following items: (1) Local swelling or induration; (2) Erythema 0.5 cm-2 cm around the ulcer; (3) Local tenderness or pain; (4) Local warmth; and (5) Purulent discharge (thick, opaque to white, or sanguineous secretion)
1	Small, shallow ulcer(s) on the distal leg or foot; no exposed bone, unless limited to the distal phalanx	0.6-0.79	70-100	40-59	Local infection involving only the skin and the subcutaneous tissue exclude other causes of an inflammatory response of the skin (*e.g.*, trauma, gout, acute Charcot neuro-osteoarthropathy, fracture, thrombosis, and venous stasis)
2	Deeper ulcer with exposed bone, joint, or tendon generally not involving the heel; shallow heel ulcer without calcaneal involvement, gangrenous changes limited to digits	0.4-0.59	50-70	30-39	Local infection with erythema > 2 cm, or involving structures deeper than skin and subcutaneous tissues (*e.g.*, abscess, osteomyelitis, septic arthritis, and fasciitis), and no systemic inflammatory response signs
3	Extensive, deep ulcers involving forefoot and/or midfoot; deep, full-thickness heel ulcers with or without calcaneal involvement, extensive gangrene involving the forefoot and/or midfoot; full-thickness heel necrosis with calcaneal involvement	≥ 0.39	< 50	< 30	Local infection with signs of SIRS, as manifested by two or more of the following: (1) Temperature > 38 °C or < 36 °C; (2) Heart rate > 90 beats/min; (3) Respiratory rate > 20 breaths/min or $PaCO_2$ < 32 mmHg; and (4) White blood cell count > 12000 or < 4000 cu/mm or 10% immature bands

ABI: Ankle-brachial index; ASP: Ankle systolic pressure; TcPO2: Transcutaneous oxygen pressure; SIRS: Systemic inflammatory response syndrome.

interphalangeal joints show flexion and malpositioning, leading to foot deformity[42,43]. Clinically, motor neuropathy often presents with sensory damage. The combination of motor and sensory neuropathy results in an unequal foot load and insecure gait with pain insensitivity, and the deformed joints and over-pressure-loaded plantar are constantly worn and develop hyperkeratosis over time, promoting the development of ulcers[32,43-45].

Autonomic system dysfunction is thought to be responsible for the pathogenesis of ulceration. Sweating dysfunction caused by autonomic neuropathy causes overheating of the skin through increased deeper blood perfusion, resulting in anhidrotic and fissural skin and a broken dermal barrier

and diminishing the effectiveness of the skin as a barrier against microbial invasion[32,46]. Moreover, the increased glycation of keratin aggravates the ulcers by causing the skin to become thick and squeezing the soft tissue that it covers[47].

PAD

PAD is a clinical term that is classically used to summarize the various diseases that affect the noncardiac and non-intracranial arteries and result in complete or partial occlusion of the peripheral arteries of the upper and/or lower limbs, leading to tissue ischemia and blood supply insufficiency[48,49]. PAD is another equally important contributor to neuropathy in the occurrence of leg ulcers and amputation[50]. The frequency of lower limb amputations in diabetes patients with PAD is higher than that in those without PAD, which may be related to a stronger association with DM in limbs below the knee because the arteries of the lower limbs, especially distal arteries like the dorsalis pedis artery, are mostly involved in DM[51-53]. Among DM patients with PAD characterized by occlusion of the lower limb arteries, one-third will experience intermittent claudication described as pain, cramp, and/or numbness of the affected limb, which occurs when exercising and at rest[52,54]. Long-term intermittent claudication causes progressive dysfunction and disability, and in combination with an impaired vasodilatory response to plantar pressures, it can result in critical limb ischemia, thus leading to foot ischemic ulceration and amputation[55-57].

Foot deformities

Together with neuropathy and trauma, foot deformity was reconfirmed by the Task Force of the Foot Care Interest Group of the American Diabetes Association as a most common triad of causes that interact and ultimately result in ulceration[34,58]. Common structural foot deformities include interphalangeal joint deformity, MTPJ deformity, pes cavus, and pes equinus[59]. The most prevalent and common deformity in DM patients is MTPJ deformity, including hammer-and-claw toes characterized by hyperextension of interphalangeal joints, and hallux valgus characterized by outward tilting of the first MTPJ[58,60].

At present, the specific course of foot deformities in patients with DM is not clear. The widely accepted pathogeny is associated with muscle atrophy, decreased joint mobility, and uneven force on the sole as a result of motor neuropathy[58,59,61]. In DM patients, the musculoskeletal components are destroyed, which is embodied by the atrophy of intrinsic and extrinsic foot muscle and fatty infiltration [62-64]. The atrophy of small muscles like the extensor digitorum brevis and/or interosseous muscles directly affects the stability of joints and the function of the foot by destroying the structure of joints and leading to MTPJ hyperextension and interphalangeal joints hypercurvation[33,65,66]. Moreover, because of incorrect overpressure, the mobility of joints gradually decreases, further aggravating the pressure on the bony prominences, particularly the metatarsal head[67]. Persistent exposure to repetitive and excessive pressure causes deformation of the metatarsal head, and pressures exceeding the threshold may lead to prolonged ischemia, causing the skin below to weaken and break down[68-71]. Meanwhile, blood supply recovery after ischemia caused by pressure changes can lead to reperfusion injury. These ischemia-reperfusion cycles may trigger an excessive inflammatory response, further aggravating the tissue injury, which is considered to be another cause of pressure ulcers[72,73].

Other factors also contribute to ulcer formation by increasing plantar pressure. Hyperkeratosis refers to a thickening of calluses caused by sustained increasing plantar pressure, and is a crucial factor that always precedes ulcer formation[59,74]. Callus thickening has been reported frequently in the plantar area of the metatarsal heads, the heel, and the middle of the big toe[59]. Once formed, it adds gentle but sustained pressure on the underlying soft tissue, and in combination with other pressures, it leads to the formation and rupture of ulcers[58,75]. Another common factor is pathological changes in the tendon, like an increased Achilles tendon size and abnormal tendon structure[76-78]. Thickened fascia and tendon limit joint activity and weaken ankle dorsiflexion, also accelerating the formation of ulcers[62,79].

DFU PREVENTION AND MANAGEMENT

The existing management systems for DFU have gradually expanded on the basis of the three principles established by Treves[80], namely, sharp debridement, offloading, and education. In this section, several commonly used management approaches and their applications are listed, indicating that multidisciplinary DFU care will eventually become the mainstream approach.

Preventive education

Foot care education and self-examination represent the cornerstone and the primary protective factor in DFU prevention[81]. Comprehensive foot care and intensive nursing education together with patient education are reported to be simple, feasible, and strongly effective for DFU prevention[82,83]. For physicians and/or podiatrists, periodic evaluation of arterial perfusion in patients with DM, especially those with peripheral neuropathy and/or foot deformity, which are the main predictive risk factors for

DFU, may help improve the foot condition. For medical institutions, strengthening publicity on preventive measures to improve patients' self-management is important and increasingly urgent[84]. The popularization of self-management should include multiple aspects like foot hygiene instruction, proper footwear use, skin lesion self-examination, and foot sensation self-evaluation. Guiding and encouraging patients to wash feet with water at a moderate temperature, keeping feet clean and dry, and inspecting the condition and checking the color of foot skin can help effectively avoid cracks caused by autonomic neuropathy and usual redness of the skin caused by overpressure[81]. For patients, more than improvements in self-management, regular screening for diabetes complications such as ophthalmic complications are essential and more cost-effective than no screening[82].

Debridement

Debridement can be performed by surgical and non-surgical methods, and both of them are used to remove nonviable or devitalized tissue from the wound bed to accelerate granulation tissue formation and re-epithelialization, which promote wound healing[85]. Experts have considered surgical debridement as the formation of a "new acute wound", since the nonviable tissue has to be debrided down to the bleeding tissue[33]. This mechanical separation is impossible without damaging normal tissues. The surgical removal of superficial necrotic and hyperkeratotic tissue caused by repeated pressure on the foot is essential for wound healing, and it is necessary for deep wounds with bone and soft tissue involvement. Non-surgical debridement includes autolytic debridement with hydrogels, enzymatic debridement, biosurgery, and mechanical debridement with hydrotherapy[86]. Medicinal maggots have shown the ability to remove nonviable tissue selectively and may reduce the risk of secondary superinfection[33], which may lead to a shortened period of wound-healing progression[87].

Glycemic control

The close relationship between blood glucose levels and the progression of diabetes complications has been reported extensively in the literature[88]. Intensive glycemic control in patients with DM has been reported to delay the occurrence of retinopathy, peripheral neuropathy, and nephropathy, all of which are the main risk factors for DFU, and thus show a positive correlation with wound healing. Various studies evaluated and reported the positive correlation of glycemic control and DFU outcomes[39,89,90]. Hemoglobin A1c (HbA1c) is an important clinical predictor of wound healing that shows an increase of 1% when wound healing decreases by 0.028 cm^2. In the Diabetes Control and Complications Trial, intensive glycemic control reduced the incidence of microvascular complications, including DPN, and a 1% decrease in the HbA1c level was accompanied by a 37% reduction in microvascular complications in the United Kingdom Prospective Diabetes Study[91].

Nevertheless, the definition of intensive beneficial glycemic control differs across trials and guidelines. The International Diabetes Federation recommended an HbA1c level lower than 6.5%[92], whereas the American Diabetes Association subdivided and specified the standards for older adults [93], children[94], and pregnant women[95], and recommended an HbA1c goal below 7% for nonpregnant adults[96]. One review of nine randomized controlled trials found that intensive glycemic control based on a target HbA1c level of 6% to 7.5% was associated with a 35% reduction in the risk of amputation in patients with diabetic foot syndrome[97,98].

However, the benefits and adverse effects of intensive glycemic control are still unclear[39]. Acute glycemic control did not show a relationship with the wound outcomes and amputation rate in DFU patients in most studies[98]. The intensity of glycemic control partly determines the incidence of hypoglycemia. In multiple types of studies, a significant adverse consequence of intensive glycemic control was the increasing incidence of hypoglycemia[39,99,100], so intensive glycemic control must also be accompanied by cautious monitoring[36]. However, the lack of clinical evidence and data supporting tight glycemic control should not deter efforts to achieve the target of optimal glycemic control, since it has been suggested to be the only significant tool to prevent complications in patients with both type 1 and type 2 diabetes[101].

Since uncontrolled hyperglycemia is one of the reasons why the readmission rate of DFU patients is as high as 30%, which is much higher than that of other patients, intensive glycemic control will help prevent such readmissions[102,103]. Besides, intensive glycemic control will help form a "glycemic memory" or "legacy effect", which implies that the benefits of earlier interventions are still evident while following the disease course[104].

Exercise

The effect of exercise on DFU is probably mediated by its effects on the risk factors. Exercise is reported to play a role in preventing or counteracting PAD in patients with type 2 DM[55], since regular physical activity may improve the claudication distance in PAD[50]. Moreover, exercise can disrupt the progression of DPN. Different types of exercise have significant effects on HbA1c reduction, and combined exercise is more effective in comparison with aerobic and resistance exercise[55]. In future studies, the exact relationship between exercise and DFU therapy should be determined to allow better integration of exercise into the treatment.

Offloading

Evidence-based guidelines have reported that reducing high foot pressure (*i.e.*, offloading) is the main objective and a significant prerequisite for promoting the healing effect and preventing ulcer[105,106]; this process involves offloading the affected area of the foot by redistributing extra pressure to other regions[107]. The majority of offloading device interventions are available for DFU and are divided into four categories: Casting, bracing, footwear, and walking aids[108]. In this section, four representative offloading devices will be introduced.

Total contact cast: The total contact cast (TCC) is often considered the gold standard device[86], and has been recommended by the guideline as the first-choice treatment option[106,109]. It protects the foot from further trauma and deformity, helps redistribution of excessive pressure[110], promotes tissue repair, and provides a protective load through below-knee-immobilization[111]. In comparison with some other approaches like removable cast walkers (RCWs) and therapeutic footwear, TCCs are reported to offer a better healing rate[108,112,113].

However, despite the substantial effectiveness of TCCs and their attractive characteristics for offloading interventions, their actual utilization rate is far from ideal. In a nationwide survey in the United States, only 1.7% of 858 centers considered a TCC as a the primary offloading method in DFU treatment[114]. Moreover, 45.5% of centers nationwide reported never using the TCC as an offloading modality, and 58.1% of centers did not consider TCCs as the first choice in noninfected plantar DFU treatment[114].

The low utilization rate can be attributed to a complex interplay of multiple factors. For patients with DFU, TCC is not easy to disassemble, which ensures their fixation and stability but hampers daily wound care if new pressure ulcers occur, hinders mobility, and results in inconvenient application because of the need for skilled technicians[107]. In addition, prolonged casting can cause stiffness of the muscles and atrophy of the joints[111], potentially leading to low patient acceptance. For medical institutions and physicians, the lack of awareness or familiarity with guidelines, the unpredictable efficacy, the inertia associated with previous practices, and the lack of skilled technicians may lead to a low level of TCC use.

RCW: A RCW is a removable knee-high offloading device. It offers multiple advantages, including easy removability, convenient wound assessments and care, and comfortable movement in daily life[115]. In comparison with TCCs, the most significant advantage of RCW is the reduction in time, energy, and experience needed for proper application[116], which makes it more suitable for frequent examination and nursing in cases of new ulcer occurrence and after an operation.

RCWs provide an equal level of plantar pressure and wound healing as TCCs and have emerged as a potential alternative to TCCs[117,118]. However, the convenience of removable RCWs may be obtained at the expense of healing ability. In *in vivo* studies, RCWs showed significantly lower healing ability in comparison with non-removable knee-high offloading devices like TCCs[117]. This significant difference in healing ability may also be caused by patients' different compliance levels while wearing the device, since patients' adherence to using the devices can promote healing. Under these circumstances, while the convenient application and removal is the greatest advantage of RCW, it also reduces the patients' compliance since the TCC cannot be removed by the patients themselves, while the RCW can[114]. Patients may be unwilling to wear the device at home, so the noncompliance in using the RCW directly affects the healing process[119].

Therapeutic footwear: Proper footwear has long been considered to play an important role in DFU care [120]. Therapeutic footwear is considered an effective approach for ulcer healing and has been used as a DFU-prevention strategy for decades[86,120]. It has been generally divided into several parts like a shoe, insole, and felted foam[108,111]. Typical diabetic prescription shoes usually have a deeper, looser, rocker outsole and toe box with soft support padding and can provide better accommodation for foot deformities[121,111]. Treatment with therapeutic shoes has been reported to yield reduced relapse in comparison with non-prescription shoes[122]. Forefoot offloading shoes (FOS) are representative prescription shoes specifically designed to offload the forefoot and have been proven to be efficacious in offloading and healing diabetic plantar forefoot ulcers. FOS mainly consist of a rocker bottom outsole and a negative-heel configuration that limits active dorsiflexion of the toes and shifts weight-bearing proximally, redistributing the load of the forefoot[107]. In comparison with standard prescription shoes, FOS reduce forefoot peak pressure ranging from 15% to 20%[123] and are recommended after surgery to offload the forefoot in case of injuries and ulcers. However, the negative-heel rocker-outsole design of FOS may compromise gait symmetry and stability, potentially decreasing wearing comfort and clinical acceptance[124,125].

Insoles have been reported to show good results in reducing shear or side-to-side stresses on the foot plantar surface, which is another key factor in DFU prevention[126]. Shear-reducing insoles are similar to dynamic foot orthosis (DFO) insoles. These insoles are composed of a free-floating distal segment and anterior segment that slide over each other[127]. This special structure is designed to reduce the shear stress on both the foot and insole. Meanwhile, a reduction in the midfoot temperature increase was observed after using DFO insoles, and since a regional foot temperature increase is associated with ulcer

formation, these findings demonstrated the protective effects of DFO insoles in DFU formation.

As one of the most commonly used accommodative dressings, the combination of felted foam with other therapeutic footwear is considered a promising approach to promote ulcer healing. Zimny *et al* [128] evaluated the effect of felted foam on wound healing in comparison with classical pressure-reducing devices and confirmed its promoting effect. Nubé *et al*[129] found that felt deflective padding applied to both skin and shoes provided similar wound-healing promoting effects for small, primarily neuropathic ulcers. Felts of different materials also influenced the healing of wounds. Pabón-Carrasco *et al*[130] reported that a combination of latex-wool felts showed great pressure-reducing ability, potentially combining wool's timely pressure capacity and latex's durability and structural stability. In comparison with wool, polyurethane, and latex, latex-wool felts offer the comprehensive advantages of hybrid materials and can serve as a great substitute for single material like wool.

In conclusion, published studies recommend the use of unremovable devices like TCCs for DFU offloading. When unremovable devices are unsuitable because of social, economic, and/or patient psychological factors and acceptance, removable devices like RCW can be used to address treatment adherence since they have the same level of therapeutic effect as unremovable devices[131]. For physicians, when choosing therapeutic footwear to assist therapy, more consideration and analysis should be paid to the specific offloading location of the foot and adherence to using offloading devices clinically[132].

Surgery

Deformities that develop into DFU commonly include hammertoes, prominent metatarsal heads, and hallux limitus[133]. A fixed-location high plantar pressure caused by structural deformities can be a predisposing risk factor for DFU recurrence if it is not adequately offloaded by the abovementioned conservative non-surgical offloading approaches. In such cases, foot surgery to ameliorate the overpressure through structural reorganization or removal of the underlying bony prominences is essential[134]. For patients showing chronic deformities and ulcers, foot surgery interventions are an important component in the management of foot ulcers, and can help them get rid of wearing cumbersome braces or footwear[133].

The offloading surgeries identified in IWDGF predominantly include tendon procedures such as toe flexor tenotomy and Achilles tendon release, but other types of surgeries can also be performed to relieve plantar pressure. Foot surgery has been classified into different types on the basis of the clinical conditions. Armstrong *et al*[135] revised a foot surgery classification system based on the presence of open wounds and acuity, and the conceptual framework of the surgery definitions in their study was based on the risk of high-level amputation. This system classifies foot surgery into four classes: Class I refers to elective surgeries aimed at reconstructing a deformed foot for patients without neuropathy, class II refers to prophylactic surgery aimed at reducing the risk of recurrent ulceration for patients with neuropathy but no open wound, class III refers to curative surgery aimed at offloading the overpressure caused by bony prominences and draining the underlying abscesses for patients with open wounds, and class IV refers to emergent surgery aimed at controlling infections caused by wet gangrene, necrotizing fasciitis, *etc.* for patients with severe infections[135].

Ahluwalia *et al*[136] systematically analyzed and summarized the five discrete types of offloading surgeries usually employed in cases of recalcitrant ulcers: (1) Lesser toe tenotomies, which aim to release the tight flexor tendon and decompress a flexible hammer toe for patients with recalcitrant ulcers on the tip or the knuckle of a deformed toe; (2) Achilles tendon release and metatarsal offloading, which aim to promote ulcer healing by releasing the Achilles tendon, metatarsal head resection(s), or joint arthroplasty; (3) Hallux procedures, which aim to redistribute the forefoot pressure by resetting the first metatarsal-phalangeal or partly amputating the hallux; (4) Surgical mastectomy, which aims to offload the overload area by directly removing the bony prominences in patients with a stable, inactive Charcot deformity; and (5) Complex surgical foot reconstruction, which aims to build a stable foot structure that can help patients walk normally without pressure areas[136].

Regular postoperative care is another extremely important aspect influencing ulcer recurrence and prevention of amputation. The reported complications after exostectomy include wound non-healing, wound dehiscence, and skin and soft tissue infection, all of which will increase ulcer recurrence and amputation rates[137]. In this regard, 70% of DFU patients have been reported to show a second ulcer recurrence after discharge, directly leading to amputation[32]. Therefore, meticulous wound care, adequate nutrition, and appropriate post-care management are essential for patients presenting with DFU, especially those who have undergone foot surgery.

Infection control

The bacterial toxins in wounds can cause infection, leading to collagen degradation, stress, and malnutrition and thereby preventing wound healing, which is a known predictor of poor prognosis and amputation[138]. Thus, correct identification and appropriate control of infections is essential to improve the prognosis in patients with DFU[86]. Diabetic foot infection (DFI) is particularly difficult to manage because the absence of exact markers to measure the level of microbiological activity for a typically colonized wound forces diagnosis based on clinical judgment[139], which often depends on the characteristics of inflammation such as per ulcer redness or induration and increased purulent

drainage[140].

In the early stage, DFU usually shows monomicrobial infections, while polymicrobial infections are observed in the middle-to-late stages[141]. Polymicrobial infections and their interactions in the DFU can delay or even stop wound healing[142]. Current clinical guidelines recommend systemic antimicrobial therapy for patients with DFI[85,139], and the formulation of a specific medication regimen is important in this regard. In the guideline developed by the Infectious Diseases Society of America (IDSA), the antibiotic regimen usually depends on the degree of infection, *e.g.*, using antibiotics targeting aerobic Gram-positive cocci for patients with mild-to-moderate infections and broad-spectrum empirical antibiotic therapy for patients with severe infections[85]. The appropriate use of antibiotics plays an important role in the prognosis of DFU, and improper or excessive antibiotic usage may cause several side effects like antibiotic resistance. IDSA advised that to avoid the adverse consequences of antibiotic overuse, narrow-spectrum antibiotics should be used for clinical treatment over the shortest term possible and discontinued immediately after the symptoms have been resolved[85].

Assessment of life quality

To avoid problems with treatment acceptance and compliance, the treatment of DFU should not only be limited to objective medical evaluation but should also include consideration of the patients' subjective feelings[143]. Assessment of the health-related QoL of patients is becoming steadily more important, especially in the treatment and evaluation of chronic diseases with a high prevalence, and should be an integral part of clinical evaluations of the prognosis of diabetes and its complications. All aspects, including physical health, pain, difficulty with usual activities, social function, role emotional, *etc.*, should be considered when evaluating the prognosis of a patient[144]. In DM patients, reductions in QoL will worsen in the presence of complications[144] such as DFU since these complications can limit physical functions such as mobility and cause pain, thereby increasing the psychological burdens caused by limitations in social relationships and fear of amputation, reducing patients' compliance with treatment, and eventually decreasing the survival rate[145-147].

Different treatment measures have shown different effects on patients' QoL. The chronicity of DM causes patients to show a higher possibility of developing psychological disorders, which is more obvious in patients who have undergone a major amputation[148]. Moreover, studies have reported significantly worse stress readaptation and deterioration of glycemic control after amputation[149], which reduces patients' QoL and weakens their socio-economic status[144]. Physical activity and exercise were confirmed to effectively improve DFU-related psychological pressure. One study reported improvements in glucose control, balance, neuropathic symptoms, and QoL of patients with DPN after Tai Chi exercises[150]. In combination with other related studies, these findings showed that patients in exercise programs have better QoL in terms of physical fitness, social ability, and emotional pressure [151].

Since offloading devices are one of the commonly used treatment modalities for DFUs, the differential influence of different types of devices on QoL should be considered clinically[152]. Although offloading devices redistribute plantar pressure and improve foot health, the accompanying adverse effects on gait and mobility should not be underestimated. Therapeutic footwear, especially when used on only one side, will cause the patient to limp while walking, causing deterioration of gait speed and symmetry, stride length, and the gait cycle time of patients with DFU. To reduce the related gait disorders and improve the patients' QoL, the use of bilateral therapeutic shoes instead of unilateral shoes can be a better option[153]. Casts show a good therapeutic effect because of their sealing ability and protective effects on wounds, which may be the reason for the higher cure rate of TCC in comparison with standard treatments[154,155]. However, the low patient acceptance of TCC is because of the limitations that it imposes on daily activities, as well as the difficulties in wound care and observation[113]. In contrast, the easy disassembly of RCW makes wound care and daily activities much more convenient, making it more acceptable for patients with DFU[114].

The QoL associated with a treatment method determines the extent to which it will be accepted and used by patients and should be one of the basic considerations when choosing therapeutic options. Currently, differences in QoL associated with different therapies have not received much attention, judging from the limited research on the relevant aspects and guidelines[156]. More studies should focus on QoL assessments to help formulate more reasonable clinical treatment plans.

CONCLUSION

DFU is a common and growing problem worldwide. The treatment approach for DFU depends on a combination of various factors that have been listed and discussed in this article. The following aspects should be considered to prevent ulcer progression and promote ulcer healing: (1) Choosing a proper classification to summarize the clinical details for further management and for auditing clinical outcomes; (2) Investigating risk factors that may predict the occurrence and promote the progression of ulcers; and (3) Employing validated interdisciplinary DFU management and care pathways, and emphasizing the cultivation of patient compliance. The findings highlight the need for the development

and application of more relevant prevention and treatment measures in the clinical management of DFU.

ACKNOWLEDGEMENTS

We thank our colleagues at the Key Laboratory of Acupuncture and Medicine Research of Ministry of Education in Nanjing University of Chinese Medicine for their support in the preparation of this manuscript.

FOOTNOTES

Author contributions: All the authors contributed to the initial writing and have read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 81873238 and 82074532; the Open Projects of the Discipline of Chinese Medicine of Nanjing University of Chinese Medicine supported by the Subject of Academic Priority Discipline of Jiangsu Higher Education Institutions, No. ZYX03KF012; and the Postgraduate Research & Practice Innovation Program of Jiangsu Province, No. KYCX22_1963.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1066-1095

DOI: 10.4239/wjd.v13.i12.1066

ISSN 1948-9358 (online)

REVIEW

Mesenchymal stem cell-derived exosomes: The dawn of diabetic wound healing

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Roncalli J, France; Shalaby MN, Egypt

Received: September 29, 2022
Peer-review started: September 29, 2022
First decision: October 21, 2022
Revised: November 4, 2022
Accepted: November 23, 2022
Article in press: November 23, 2022
Published online: December 15, 2022

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Abstract

Chronic wound healing has long been an unmet medical need in the field of wound repair, with diabetes being one of the major etiologies. Diabetic chronic wounds (DCWs), especially diabetic foot ulcers, are one of the most threatening chronic complications of diabetes. Although the treatment strategies, drugs, and dressings for DCWs have made great progress, they remain ineffective in some patients with refractory wounds. Stem cell-based therapies have achieved specific efficacy in various fields, with mesenchymal stem cells (MSCs) being the most widely used. Although MSCs have achieved good feedback in preclinical studies and clinical trials in the treatment of cutaneous wounds or other situations, the potential safety concerns associated with allogeneic/autologous stem cells and unknown long-term health effects need further attention and supervision. Recent studies have reported that stem cells mainly exert their trauma repair effects through paracrine secretion, and exosomes play an important role in intercellular communication as their main bioactive component. MSC-derived exosomes (MSC-Exos) inherit the powerful inflammation and immune modulation, angiogenesis, cell proliferation and migration promotion, oxidative stress alleviation, collagen remodeling imbalances regulation of their parental cells, and can avoid the potential risks of direct stem cell transplantation to a large extent, thus demonstrating promising performance as novel "cell-free" therapies in chronic wounds. This review aimed to elucidate the potential mechanism and update the progress of MSC-Exos in DCW healing, thereby providing new therapeutic directions for DCWs that are difficult to be cured using conventional therapy.

Key Words: Diabetic wounds; Wound and injuries; Mesenchymal stem cells; Exosomes; Pre-conditioning; Preclinical translation

Core Tip: Diabetic chronic wounds (DCWs) are one of the most serious chronic complications of diabetes, and the efficacy of stem cell therapies for refractory chronic wounds has been studied previously. Stem cell-derived exosomes are one of the important active components of stem cell paracrine secretion, which inherit the wound repair capacity of parental cells as parts of novel cell-free therapies in addition to cell-bases ones. Herein we discuss the mechanism and latest progress of mesenchymal stem cell-derived exosomes in promoting DCW healing.

Citation: Wu J, Chen LH, Sun SY, Li Y, Ran XW. Mesenchymal stem cell-derived exosomes: The dawn of diabetic wound healing. *World J Diabetes* 2022; 13(12): 1066-1095
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1066.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1066

INTRODUCTION

Wound healing after skin tissue injury relies on a dynamic chain of physiological reactions including hemostasis, inflammation, cell proliferation, and tissue remodeling[1]. Any step out of balance, such as excessive inflammation, impaired fibroblast migration and proliferation, abnormal collagen formation and deposition, and hindered re-epithelialization, ultimately leads to delayed wound healing and formation of chronic wounds. Chronic wounds are those that have failed to proceed through an orderly and timely reparative process to produce anatomical and functional integrity of the injured site[2]. They refer to wounds caused by multiple factors that have not healed or have not demonstrated a tendency to heal after a certain period clinically, with a chronic duration ranging from 4 to 12 wk[3,4]. Various pathological states result in chronic wound development, including diabetes, pressure injuries, infections, and arterial/venous insufficiency of which reports are similar in China and developed Western countries[4-6], which have the most complicated pathogenesis and therapeutic strategies being diabetic chronic wounds (DCWs).

Diabetes mellitus (DM) is a metabolic disease characterized by elevated blood glucose levels, of which DCWs are among the most threatening complications. The combination of a high-glucose environment and several biological factors, including ischemia and hypoxia, abnormal inflammatory response, excessive oxidative stress, and peripheral neuropathy, contributes to wound formation[7-9]. Such wounds have problems of protracted healing, long treatment time, difficulties in management, high cost, repeated attacks, and high disability/mortality rates, resulting in heavy physical, psychological and economic burdens[10,11]. The intervention of DCWs cannot be underestimated based on what is mentioned above. Hence, solving persistent inflammation, impaired cell proliferation and migration, decreased angiogenesis, and remodeling of the extracellular matrix (ECM) is important. Innovative wound repair methods, such as local negative pressure, growth factors, and autologous platelet-rich gels, have remarkable effects on healing DCWs[12-15]. However, more specific treatment options are required for refractory and contraindicated wounds.

With the rapid development of tissue engineering, cell therapies have gradually become widely used in various disciplines. Stem cells can be used in regenerative medicine and play an indispensable role in wound repair[16], of which mesenchymal stem cells (MSCs) are the most commonly used. MSCs have self-renewal abilities and multi-directional differentiation potential, participating in damage repair through intercellular communication and bioactive factor secretion, finally achieving the effect of promoting wound healing[17]. Clinical trials of MSCs for treating various types of cutaneous wounds are currently in full swing, and their efficacy and safety in promoting wound regeneration have been initially demonstrated. As clinical trials continue to progress, further attention and supervision need to be paid to their potential safety issues of proliferative lesion formation, abnormal organ reaction and unknown long-term health effects after transplantation[18-20].

Studies have revealed stem cells promote repair and regeneration mainly through paracrine signaling, whereas exosomes are one of their important paracrine active components[21]. MSC-derived exosomes (MSC-Exos) carry genetic information, functional RNAs, and proteins from parental cells, demonstrating wound healing effects *via* intercellular communication after these biologically active substances are acquired by recipient cells[22-24]. Thus, MSC-Exos have broad application prospects in diabetic wound repair[25]; however, they have not yet been carried out in clinical practice. The important role of MSC-Exos in all stages of diabetic wound healing and the preclinical application are highlighted in this review, to pave the way for their use as an effective tool in the management of these harmful diabetic complications.

DCWS: HEALING DISORDERS CAUSED BY VARIOUS MECHANISMS

DM is a metabolic disease characterized by elevated blood glucose levels, which poses a serious threat to human health. The continuous progression of hyperglycemic toxicity without effective control will affect macrovascular, microvascular, and peripheral nerves throughout the body and involve various organs such as the brain, eyes, heart, kidney, and skin, resulting in various diabetic chronic complications[26]. DCWs are one of the most common and threatening chronic complications, often accompanied by infection or deep-tissue destruction[27]. Protracted wounds are the most common cause of non-traumatic amputations. Diabetic foot ulcers (DFUs) are characterized by wounds on the feet, which are the most typical, and patients with DFUs have a 2.5 times higher risk of 5-year mortality than those with none[28]. The overall mortality of DFUs within 5 years is nearly 50%[29], and approximately 20% of moderate-to-severe DFUs will lead to amputation; the 5-year mortality rate after amputation exceeds 70%[30].

Impaired wound healing processes caused by hyperglycemia-induced disturbances in wound-linked cellular behaviors contribute to diabetic wound healing difficulties[7,31]. Hyperglycemia, oxidative stress, and insulin resistance affect the function of vascular smooth muscle cells, endothelial cells, and platelets, which in turn may lead to abnormal coagulation processes and affect platelets of triggering for subsequent inflammatory and proliferative phases[32]. The hyperglycemic microenvironment can lead to dysfunction of immune and inflammatory cells and dysregulation of inflammatory factors. Perpetuated inflammatory states induced by increased mast cell degranulation[33], excessive extracellular traps produced by neutrophils[34], dysregulated and persistent M1 (pro-inflammatory) macrophage polarization[35], pro-inflammatory factors (IL-1β, TNF-α, and IL-6) overexpression, and anti-inflammatory factors (IL-10 and TGF-β) deficiency finally hinder wound healing[7]. The proliferative phase of diabetic wound healing is characterized by disturbed physiological functions of keratinocytes[36], fibroblasts[37], and endothelial cells[38], then the impaired re-epithelialization, granulation tissue formation, matrix deposition, and angiogenesis affect wound healing. Various factors also affect the function and activity of these cells during this phase, including decreased chemokines with pro-angiogenesis produced by macrophages, hemoglobin glycation, vascular stenosis, increased oxygen consumption affecting oxygen-dependent cellular behaviors, and impaired nerve fiber regeneration[7,31,39,40]. Remodeling of the ECM spans the entire injury response, and fibroblasts are the major cell type responsible for this phase[31]. Sequential changes in the ECM require a balance between collagen degradation and synthesis, achieved through temporal regulation of the dynamic changes in the ratio of matrix metalloproteinases (MMPs) to tissue inhibitors of metalloproteinases (TIMPs)[41,42]. Such changes in DCWs are unbalanced and lead to difficult wound healing and excessive scarring[41,43]. However, no clear demarcation exists between the various stages of wound healing, and functionally impaired cells can interact, eventually leading to poor diabetic wound healing, progressing to local infection, gangrene, and even amputation. Therefore, the most important aspect of effectively treating DCWs is to identify an appropriate approach that can comprehensively improve abnormalities in all phases of wound healing.

CURRENT STRATEGIES AND PROMISING DIRECTIONS FOR DCWS REPAIR

Traditional strategies for DCWs management include glycemic control, conventional dressings (*e.g.*, hydrocolloids, alginates, and silver ions, *etc.*), thorough debridement (*e.g.*, surgical, mechanical, ultrasonic waterjet, collagenase, and maggot, *etc.*), wound off-loading, autologous skin and skin substitute grafting, infection control, and revascularization, *etc.* These strategies are used to create the wound bed microenvironment suitable for repair through moisture balance maintenance, necrotic or inactivated tissues removal, systemic and local infections control, and local blood flow improvement[13,44-46]. Negative pressure wound therapy can also be used to achieve its role in improving wound exudate drainage, enhancing local perfusion, removing bacterial products, promoting granulation tissue growth, and facilitating wound healing[47]. However, these conventional treatments are often ineffective in many patients because of impaired cell function around the wound sites caused by underlying microenvironmental alterations[48]. Several innovative wound adjuvant therapies, including exogenous supplementation of growth factors[49], platelet-rich plasma[50], autologous platelet-rich gels[15,51], and hyperbaric oxygen therapy[52] have been developed to promote the activity and function of damaged cells and offer the possibility of treating unselected refractory wounds. However, an updated systematic review has revealed that some measures had positive effects on accelerating wound healing, while others had limited impacts on diabetic ulcer healing[53]. However, the overall efficacy of various treatment modalities for DCWs remains unsatisfactory, and effective therapeutic strategies need to be continued.

STEM CELL-BASED THERAPIES BECOME HOT TOPICS, COMING EXOSOMES INTO BEING

Stem cells have the potential for self-renewal and multidirectional differentiation with great research and application value in life sciences, clinical trials and disease research. Stem cell-based therapies are now approved by several countries, and have been widely used in various disciplines. MSCs are currently the main experimental cell sources and have shown their excellent therapeutic potential and value in clinical trials in the field of regenerative medicine[16,54].

MSCs provide assistance in all phases of wound healing by exerting their functions of regulating skin homeostasis and wound healing through migration into the skin damage site and interaction with skin cells and can influence the function of these cells by paracrine secretion of bioactive factors and differentiation into them[55,56]. As MSCs have exhibited wound healing in many preclinical studies as powerful tools for regulating inflammation, promoting cell proliferation and migration, angiogenesis, and collagen synthesis[57-60], the application of MSCs for DCWs contributes to progress toward clinical trials. Twenty-five clinical trials of MSCs for diabetic ulcers have been conducted or are recruiting subjects, which are recorded in the ClinicalTrials.gov database (clinicaltrials.gov).

Previous clinical studies have demonstrated that MSC transplantation in patients with DFUs is safe and feasible with the properties of improving microcirculation, wound healing, ulcer recurrence, and amputation[61-63]. However, stem cell therapies are still in their early clinical stage, further attention and supervision are required of declined performance during production and application as cellular senescence and loss of multipotency during *ex vivo* expansion and from variable donors[64,65], decreased survival rate caused by advanced glycosylation end products[66], potential safety issues as proliferative lesion formation and abnormal organ reaction[20], and unknown long-term health effects after transplantation. Basic and clinical researches related to allogeneic/autologous stem cells are subject to the International Society for Stem Cell Research Guidelines for Clinical Translation of Stem Cells and national ethical guidelines and related guidelines/regulations[20,67].

MSCs exert their repair and regenerative effects mainly through paracrine signaling, and exosomes are one of the important active components[21] that provide a more stable entity that minimizes the potential safety concerns for cell transplantation. MSC-Exos play an important role in intercellular communication by carrying various important functional substances of parental cells, being used of promoting wound healing[68,69]. Compared to direct cell transplantation, MSC-Exos avoid the immune rejection because of low immunogenicity; allow to cross various biological barriers and avoid the risk of embolism from intravenous injection based on their smaller sizes[70]; the dose and fraction can be adjusted artificially and genetic modifications are easier and safer[71]; avoid the problem of malignant transformation; and allow to repair diabetic complications through multiple actions[72]. They can also be used as ideal carriers for carrying and delivering therapeutic drugs, genes, enzymes, or RNAs[73], and their efficiency and targeted transport capacity can be tuned through pretreatment or engineering transformation[74], demonstrating their promising applications in the field of repair and regeneration.

STEM CELL-DERIVED EXOSOMES: NOVEL CELL-FREE STRATEGIES

Exosomes biology

The concept of "exosomes" was first proposed in 1981 by Trams *et al*[75], using to collectively refer to extracellular vesicles (EVs) that originated from the exudation of various cell line cultures. The currently defined exosomes were first discovered in sheep reticulocytes and considered cellular waste[76-78]. Of note, "EVs" is the preferred term by the International Society for Extracellular Vesicles (ISEV) to describe all nanoparticles with lipid bilayer structures released by cells[79].

Exosomes, the biological nanoscale spherical lipid bilayer vesicles[80], can be secreted by almost all cell types and are widely present in cell culture supernatants and many body fluids[81]. Their diameters range from 10 to 200 nm. In addition to exosomes, EVs also include microvesicles that are also called ectosomes with a diameter of 100-1000 nm, and apoptotic bodies larger than 1000 nm according to different sizes and biogenesis[82,83]. The types and functions of the bioactive substances carried by exosomes differ according to their cellular origins and adjacent cellular components[84]. The major substances include genetic information, RNA species (mRNA, tRNA, rRNA, miRNA, lncRNA, circRNA, *etc*.), proteins, lipids, cytokines, and growth factors[85,86]. Exosomal proteins include intrinsic components involved in exosome biogenesis, such as fusion-related proteins (GTPases, annexins, flotillin, and Rab proteins), heat shock proteins (HSP70 and HSP90), tetraspanins (CD63, CD81, CD82, and CD9), ESCRT complex, and specific functional proteins originating from parental cells[87]. Apart from serving as a medium for cellular communication, some proteins are also involved in the membrane composition and biosynthesis as identified biomarker proteins and can provide stability and permeability in concert with phospholipid bilayers.

Exosomes originate from endosomes during generation, circulation, degradation, and liberation[88]. Extracellular substances fuse with early sorting endosomes through plasma membrane invagination and

endocytosis, and begin to accumulate bioactive substances. Eventually, they mature into late sorting endosomes, which invaginate to form intraluminal vesicles that can then generate multivesicular bodies (MVBs)[68,88]. MVBs can be absorbed by lysosomes comprising a degradative pathway, or they can undergo a specific exocytotic process whereby they fuse with the plasma membrane to release exosomes into the extracellular space[89]. After release, they act as mediators of intercellular and intra-organ communication to transfer the contained bioactive substances to recipient cells through direct fusion, endocytosis, and receptor-ligand binding to affect their functions[90,91], participating in the body's physiological and pathological state adjustment[92].

Isolation and characterization of exosomes

The extraction of exosomes is primarily based on their physicochemical properties. This process is difficult because of the heterogeneity of exosomes derived from different cell origins, the possible existence of subpopulations of exosomes with different functions and phenotypes even when extracted from a single cell line, and multiple EV subtypes with similar biophysical properties[93]. Therefore, different isolation methods should be targeted for different purposes[87]. Differential ultracentrifugation is the most widely used separation technique and is also known as the gold standard for isolation, while the main principle is to harvest the desired components based on size and density differences[94]. Polymer precipitation uses polyethylene glycol to harvest exosomes under centrifugal conditions by reducing their solubility[95]. Size-exclusion chromatography[96] and ultrafiltration[97] are both based on size differences between exosomes and other components, although they may adulterate other particles of similar size. Immunoaffinity capture is based on the specific binding of antibodies and ligands to isolate exosomes from a heterogeneous mixture[98]. Current isolation and purification techniques have varying effects and many problems such as low purity and recovery, structural damage, and time and cost consumption, making achieving efficient enrichment difficult, which has become a bottleneck of the translational applications of exosomes[87]. Hence, continuously exploring new isolation and purification techniques or combining multiple techniques is necessary to improve the isolation efficiency and thus obtain ideal exosomes.

Exosomes are mainly characterized by external characteristics (morphology and size detection) and the identification of surface markers[87]. As mentioned above, some protein components of exosomes serve as surface protein markers for identification. The ISEV has proposed the need to identify two types of proteins as follows: one is the biomarker proteins shared by exosomes to determine whether the extracted components are exosomes, and the other is cell-type-specific exosomal proteins that need to be identified to determine cellular origin[79]. Therefore, exosomes can be characterized by detecting their morphology using transmission electron microscopy, their size and concentration by dynamic light scattering, and nanoparticle tracking analysis technology, and their marker proteins by western blot, enzyme-linked immunoassay, and flow cytometry[87].

Biological functions of MSC-Exos

Stem cells have self-renewal abilities and multi-directional differentiation potential, while MSCs are one of the most frequently used and promising adult stem cells that can be derived from most adult tissues such as the bone marrow, adipose tissue, and umbilical cord[99,100]. Bone marrow-derived MSC-Exos (BMSC-Exos) are biologically stable, have low immunogenicity, and exhibit good proliferation and viability after transplantation. They are most commonly used in clinical trials and can play a prominent role in various disorders, especially bone-related diseases[101]. Umbilical cord-derived MSC-Exos (UCMSC-Exos) can be isolated non-invasively, with low immunogenicity and strong self-renewal and proliferation ability, although it has limitations in maintaining bioactive and clinical therapeutic transport[102]. Adipose-derived MSC-Exos (AMSC-Exos) have relatively abundant sources that can be easily obtained by painless minimally invasive surgery; they are also pluripotent, plastic, easy to store, and stable in blood or body fluids[103]. Exosomes of different origins share most of their bioactive factors and are generally similar in their biological functions; however, their specific biological properties depend on the molecules that are specifically expressed[104].

MSC-Exos are involved in intercellular communication through the transfer of proteins, RNA, DNA, and bioactive lipids that can be delivered to target cells to regulate their activities and functions[68]. They are generally involved in the regulation of cell survival and differentiation, the immune system, and inflammation modulation, and are also capable of promoting angiogenesis and tissue remodeling [73]. Considering these multiple biological functions, several studies have also reported that the MSC-Exos play a therapeutic role in autoimmune diseases[105], ischemic injuries[106], and metabolic diseases [107], and are also related to dynamically modulating tumor biological functions[108], promoting repair and regeneration of damaged osteochondral, neural, and tendon tissues, and facilitating wound healing [109-112]. Current studies also discovered that they can improve COVID-19-related cytokine storms and the deterioration of lung function due to severe pneumonia[113].

MSC-EXOS FOR REPAIRING DIABETIC WOUNDS

MSC-Exos play an important role in each phase of wound healing[81]. They can regulate diverse cell types related to wound repair by enhancing or suppressing certain bioactivities, achieving hemostasis, inflammatory regulation, cell migration to the wound site, cell proliferation, and differentiation to form granulation tissue, angiogenesis, and ECM reorganization[69]. They can also be expected to be therapeutic agents for different types of diabetes by alleviating autoimmune damages[114], attenuating insulin resistance, and improving β-cell exhaustion[115]. Additionally, they can be used to prevent and treat DM-related complications. Based on these potentials, MSC-Exos may be of considerable importance in DCW treatment.

Hemostasis

Tissue factor (TF) is an initiator of coagulation activation and was identified in the plasma membrane of exosomes[116]. TF can transfer to the platelets and initiate the extrinsic coagulation cascade, leading to the conversion of prothrombin to thrombin and fibrin clot formation[117]. Induced coagulation and stimulated thrombogenicity were observed using EVs carrying TF from the pericardial blood of patients who received cardiac surgery[116]. Rat BMSC-Exos were applied to the bleeding site in the hemorrhage liver model, which exhibited an inhibited amount of bleeding and shortened bleeding time, demonstrating their excellent hemostatic properties. However, no studies related to exosomes' promotion of coagulation in cutaneous wound healing have been conducted. Further studies are needed to demonstrate the potential role of exosomes in the hemostasis phase of wound healing.

Inflammation

Excessive inflammation is a major cause of persistent diabetic wounds. Abnormal macrophage polarization and cytokine overexpression lead to an uncontrolled and persistent inflammatory state and can cause secondary tissue damage[7]. MSCs-Exos can inhibit the differentiation, activation, and proliferation of T cells as well as reduce IFN-γ release[118]. They can reduce the concentration of the inflammatory cytokines, TNF-α, iNOS, IL-1β, and IL-6[119] and upregulate the expression of the anti-inflammatory cytokine IL-10[120,121]. MSCs-Exos can also induce M2 polarization of macrophages to promote wound healing by delivering exosome-derived miR-223 to target regulating the expression of pknox1 protein[122].

Such abilities can also be observed in diabetic wounds. Topical application of native AMSC-Exos to diabetic mice dorsal full-thickness skin wounds also downregulated inflammatory cytokines (IL-6, TNF-α, CD14, CD19, and CD68) expression and promoted wound healing[123]. Similar alleviated inflammatory effects achieved by regulating inflammatory factors could also be observed in the combination of intraperitoneal Nrf2 pharmaceutical activator and BMSC-Exos subcutaneous injection, demonstrating decreased inflammatory cytokines TNF-α and IL-1β and increased anti-inflammatory cytokines IL-4 and IL-10[124]. Intradermal injection of MSC-Exos derived from human menstrual blood could induce macrophage polarization from the M1 to M2 phenotype, while this capacity is better than that of menstrual blood-derived MSCs[125]. Significantly lower M1 polarized macrophages and higher M2 polarized macrophages were also observed in the diabetic mouse air pouch model and diabetic rat full-thickness skin wound model using BMSC-Exos, while melatonin-stimulated BMSC-Exos (MT-Exos) had stronger effects[121]. Immunomodulatory capacity was enhanced after preconditioning. Moreover, MT-Exos could improve wound healing by activating the PTEN/PI3K/AKT signaling pathway to promote macrophage M2 polarization, angiogenesis, and collagen synthesis; promote the resolution of persistent inflammation; and drive the transition from inflammation to proliferation[121]. HUCMSC-Exos pretreated with lipopolysaccharides have better regulatory properties for macrophage polarization and resolution of chronic inflammation by transferring miR-let7b, while the TLR4/NF-κB/STAT3/AKT pathway is important in regulating this mechanism to promote wound healing[126]. The use of engineered TNF-α/hypoxia-pretreated HUVMSC-Exos in infected DCWs also decreased proinflammatory cytokines (TNF-α, IL-1β, and IL-6), induced M2 macrophage polarization, reduced bacterial burden, and bacterial colonization at the wound sites. Reduced levels of oxidative biomarkers and increased levels of antioxidant mediators also demonstrated the ability of oxidative stress suppression [127]. The combination of BMSC-Exos and carboxyethyl chitosan-dialdehyde carboxymethyl cellulose hydrogel revealed skewed macrophage functional polarity from M1 toward an anti-inflammatory M2 phenotype, as well as enhanced antibacterial effects by significantly inhibiting bacterial growth[128].

Proliferation

Fibroblasts, keratinocytes, and endothelial cells participate in the proliferative phase. Unlike the dual regulatory effects on the tumor, MSC-Exos directly affect the proliferative phase of wound healing by stimulating the proliferation and differentiation of these cells, as well as promoting angiogenesis at injury sites[104]. Enhanced migratory and proliferative capacity and inhibited apoptosis of keratinocytes by activating the AKT/HIF-1α and Wnt/β-catenin pathways were observed with AMSC-Exos[129,130]. BMSC-Exos demonstrated the ability to promote fibroblast proliferation, migration, and secretion of growth factors and can induce tube formation in human umbilical vein cells (HUVECs)

[131]. AMSC-Exos induced angiogenesis in both *in vivo* and *in vitro* experiments, and the promotion of angiogenesis in endothelial cells was achieved by transferring miR-125a to inhibit DLL4 expression, accompanied by the downregulation of pro-angiogenic genes (Ang1 and Flk1), and upregulation of anti-angiogenic genes (Vash1 and TSP1)[132]. In addition to its pro-proliferative ability *in vitro*, the pro-healing effect of MSC-Exos has also been observed in acute non-diabetic wounds. MSC-Exos from human umbilical cord Wharton's jelly could regulate HaCaT cell function by suppressing AIF nucleus translocation and PARP-1 hyperactivation, thus attenuating full-thickness skin wounds by enhancing re-epithelialization and angiogenesis[133]. Fetal dermal-derived MSC-Exos accelerated wound closure in a mouse full-thickness skin wound model by activating the Notch signaling pathway to promote the motility and secretory capacity of fibroblasts[134].

Similarly, exosomes from MSCs improve proliferation and angiogenesis in diabetic wounds. AMSC-Exos accelerated cutaneous wound healing in diabetic mice with full-thickness skin wounds model by enhancing cell proliferation, inhibiting apoptosis, and promoting angiogenesis. They also repaired skin barrier functions, and produced large amounts, regular arrangement, and dense distribution of new collagen[123]. Shabbir *et al*[131] have also reported that these cells significantly increased their proliferation when treated with MSC-derived exosomes. Enhanced angiogenesis and fibroblasts proliferation, migration, and differentiation abilities were observed in diabetic wounds treated with human decidua derived MSC-Exos, as well as an improved fibroblast senescent state, reduced scar width, and larger and better-organized collagen deposition[135].

Various methods have been used to modify MSC-Exos to enhance fibroblast proliferation and angiogenesis. Co-culture of lncRNA H19-transfected BMSC-Exos with fibroblasts extracted from foot tissue of patients with DFUs revealed that overexpressed exosomes regulated the PTEN-mediated PI3K/AKT signaling pathway by competitively binding miR-152-3p to enhance proliferation and migration of fibroblasts and inhibit apoptosis and inflammation[136]. Injecting such exosomes into the peri-wound tissue of diabetic mice revealed the same changes in expression and accelerated wound healing[136]. Atorvastatin-pretreated BMSC-Exos promoted proliferation, migration of HUVECs, and vascular endothelial growth factor (VEGF) expression and accelerated wound healing in diabetic full-thickness skin injury rat models[137]. Pioglitazone-pretreated BMSC-Exos-treated full-thickness wounds in diabetic rats achieved faster-wound closure, with more adequate re-epithelialization and extensive collagen deposition, significantly enhanced wound perfusion, and had significantly upregulated levels of VEGF and CD31[138]. Subcutaneous injection of mmu_circ_0000250-modified AMSC-Exos *via* miR-128-3p/SIRT1-mediated autophagy promoted wound healing in diabetic mice, and increased capillary and granulation tissue production was detected owing to promoted proliferation and migration and reduced apoptosis of endothelial cells[139].

Biological scaffolds can improve the survival of exosomes in the inflammatory environment of diabetic wounds and maintain their sustained release. UCMSC-Exos combined with the Pluronic F127 hydrogel revealed promoted chronic wound healing in diabetic mice. The elevated number of blood vessels and microvascular density, enhanced regeneration of granulation tissue, and cell proliferation were also observed, with the significant formation of new hair follicles in the center of the wounds, sufficient subepidermal collagen deposition, and orderly arrangement of collagen fibers[140]. Similar changes were observed in the wounds of diabetic mice using engineered bioactive self-healing antimicrobial exosome hydrogels (FHE@exo), and the elevated number of dermal appendages and differentiation and re-epithelialization of the epidermis were also observed[141]. The combination of human gingival tissue-derived MSC-Exos (GMSC-Exos) and a chitosan/silk hydrogel sponge promoted re-epithelialization, angiogenesis, and collagen deposition, while the increased nerve fiber density also reflected enhanced neuronal ingrowth in the proliferative stage[142].

Matrix remodeling

In the final stage of wound healing, the production and remodeling of the ECM are key factors in determining the time of wound healing and degree of scarring. Recently, some studies have reported on the effects of exosomes on matrix remodeling. BMSC-Exos have been demonstrated to restore normal skin morphology in rats with full-thickness skin injury[143], while these capacities relied on the downregulation of TGF-β1 and upregulation of TGF-β3 by inhibiting the TGF-β/Smad signaling pathway. UCMSC-Exos had large amounts of miR-21, miR-23a, miR-125b, and miR-145, while it inhibited the differentiation and excessive aggregation of myofibroblasts and exerted an anti-scarring effect *via* the TGF-β2/Smad2 pathway *in vivo*[144]. UCMSC-Exos can also promote the phosphorylation of YAP, a key site of the Hippo pathway, to negatively regulate the Wnt4/β-catenin pathway to balance tissue regeneration and repair, with excessive cell proliferation and collagen deposition in the remodeling stage[145]. It was noted that intravenous injection of ADSC-Exos could increase the ratio of type III collagen to type I and TGF-β3 to TGF-β1, prevent fibroblast-to-myofibroblast differentiation, and reduce scarring at incisions in the full-thickness skin injury models[146]. They could also induce the ERK/MAPK pathway in fibroblasts to increase the expression of MMP3, thereby increasing MMP3/TIMP1 to regulate ECM remodeling[146].

In contrast to the promoted cell proliferation and abundant granulation tissue in the early stage of healing, proliferative activities were reduced during the late repair stage to prohibit tissue hyperplasia when using FHE@exo, suggesting entry into the remodeling phase that prevents excessive tissue prolif-

eration to promote wound healing[141]. The application of GMSC-Exos with chitosan/silk hydrogel sponge on the wounds of diabetic rats revealed more collagen deposition and thick wavy collagen fibers that were arranged in an orderly fashion, which is similar to that in normal skin, implying enhanced ECM remodeling[142]. These were also observed in the local transplantation of HUCMSC-Exos with polyvinyl alcohol/alginate nano hydrogel and of miR-126-3p overexpressed synovial-derived MSC-Exos with hydroxyapatite/chitosan composite hydrogel[147,148]. Altogether, these studies indicate that MSC-Exos play a pivotal role in the ECM remodeling phase of wound healing.

The various stages of wound healing are closely interwoven. MSC-Exos inherit the genetic information of their parental cells and can transfer the therapeutic bioactive substances to target cells to participate in intercellular communication, resulting in the regulation of target cell function and promotion of wound healing[81,149]. We analyzed the current preclinical application of MSC-Exos in diabetic wound models, and the cell source, administration method, dose, frequency, animal type, wound diameter, efficacy, and possible molecular mechanisms are summarized in Table 1[104,121,123-128,147,148,135-142,150-158]. Additionally, MSC-Exos were not only responsible for a specific stage but also promote microenvironment changes in the wounds at each stage to exert a pro-healing effect. Although the biological functions of promoting diabetic wound healing are generally similar, certain differences exist in the regulated signaling pathways of different cell-derived exosomes or receiving different preconditioning, according to previous studies. The regulatory mechanisms most frequently studied in diabetic wound models and may potentially confirmed in DCWs, as well as the microenvironmental changes in inflammatory and proliferative stages of wound healing after using MSC-Exos, are depicted in Figure 1.

CURRENT STATUS AND PROSPECTS OF CLINICAL APPLICATIONS OF EXOSOMES IN DCWS

Preclinical studies have demonstrated the ability of MSC-Exos to promote diabetic wound healing. No evident pathological abnormalities in the heart, liver, spleen, lung, and kidneys sampled after exosome treatment were observed, and biomarkers reflecting liver and kidney function blood biochemistry were also within normal limits[127]. Meanwhile, no erythema, edema, or irritation was observed in the wound area after exosome treatment[137], confirming the superior biosafety of exosome therapy.

We also searched for applications of exosomes secreted by stem cells from other sources in diabetic wounds and summarized them in Supplementary Table 1. Noteworthy, the types of animals used for modeling were limited to mice and rats. Most of the studies involved acute diabetic wounds, that is, exosomes were administered immediately after successful modeling of full-thickness skin wounds. Only one study introduced *Staphylococcus aureus* to establish infected chronic wounds after the establishment of full-thickness cutaneous wounds and confirmed that exosomes were effective in treating infectious DCWs[127]. The efficacy and safety of MSC-Exos need to be further confirmed in larger animal models and DCW models. Because the islet morphology, structure and function, blood biochemical indices, and skin structure of minipigs are more similar to those of the human body, they are ideal animal models for studying diabetic wounds[159]. Our team has established a chronic skin ulcer model in diabetic miniature pigs in the early stage[160] and is researching on exosome products to explore the optimal administration methods and dosages and to verify their therapeutic effects.

According to the search results in ClinicalTrials.gov, no clinical trials of MSC-Exos and exosomes from other sources for diabetic cutaneous wound healing have been registered. Therefore, we expanded the scope of clinical trials to search for exosomes derived from any sources and exosome-enriched stem cell-conditioned medium in various wound types (Table 2). None of the included four registered clinical trials had related results published, while they were all non-randomized one-arm pilot studies. Thus, more high-quality randomized controlled trials are required to further confirm these research results. Of note, the application of cell-free therapies in clinical patients requires special attention to security, although no adverse reactions of exosomes have been reported in preclinical studies. Moreover, ADSC-Exos has been confirmed to not induce any irritation or toxicity in skin sensitization, irritation, or oral toxicity tests[161]; therefore, they can be considered in clinical practice to promote wound healing in combination with basic wound care measures. Nevertheless, toxicological analysis of different tissue-derived MSCs-Exos and more evidence of short and long-term health safety assessments are required to confirm their safety.

Exosome research is still in its infancy, and the realization of the transformation from preclinical research to clinical application still has great exploration value. The problems of optimal preparation, extraction, isolation, and storage of exosomes on a large scale and their production efficiency have not yet been determined; preparation and identification of components due to different source cells and the high heterogeneity of exosome components have not yet been solved; specific regulatory mechanisms in DCWs have not yet been fully elucidated; efficacy and safety of different cell sources and/or administrations have not been proven, and reasonable and effective methods of fusing exosomes with other biomaterials have not yet been implemented, all these issues are barriers that limit the clinical application of exosomes.

Table 1 Mesenchymal stem cell-derived exosomes application of diabetic full-thickness acute/chronic cutaneous wounds model

No.	Ref.	Institution(Nation)	Exosomes source	Intervention, administration, dose and time	Control	Model species	Wound diameter	Therapeutic effect	Molecular mechanism
1	Yang *et al*[140], 2020	The Third Affiliated Hospital of Southern Medical University(China)	Human umbilical cord	1 HUCMSC-Exos + PF-127 hydrogel; injected topically; 100 μg in 100 μL PF-127 (24%); at Day 0 2 HUCMSC-Exos + PF-127 hydrogel; injected topically; 100 μg in 100 μL PBS; at Day 0 3 PF-127 hydrogel; injected topically; 100 μL PF-127 (24%); at Day 0	PBS (100 μL)	Rats (Sprague-Dawley)	10 mm × 2 (1.5 cm apart)	1 Accelerated wound closure rate 2 New hair follicle formation, fibroblasts proliferation, sufficient and order collagen deposition 3 Reduced inflammatory cell infiltration 4 Higher microvessel densities and higher number of blood vessels (CD31, MVD) 5 Promoted cell proliferation (Ki67) and enhanced regeneration of granulation tissue 6 Upregulated expression of VEGF and TGF-β 7 Hydrogel supported exosome survival and biological activity	–
2	Wang *et al*[141], 2019	The Affiliated Hospital of Wenzhou Medical University; Xi'an Jiaotong University(China)	Mouse adipose tissue	1 AMSC-Exos + F127/OHA-EPL hydrogel; covered the wound; 10 μg; at Day 0 2 AMSC-Exos; covered the wound; 10 μg; at Day 0 3 F127/OHA-EPL hydrogel; covered the wound; 10 μg; at Day 0	Saline	Mice (ICR)	8 mm × 2 mm	1 Accelerated wound closure rates 2 Promoted cell proliferation and abundant granulation tissue in early stage of healing; reduced proliferative activities during the late repair stage to prohibit tissue hyperplasia 3 Abundant and well-organized collagen fibers, more collagen deposition (Col I, Col III) 4 Faster re-epithelization (cytokeratin) and epithelial cell differentiation	–

								5 Promoted angiogenesis (α-SMA) and blood vessels formation 6 Complete skin regeneration: skin appendages and less scar tissue appeared	
3	Liu *et al*[121], 2020	Second Military Medical University; Shanghai Sixth People's Hospital affiliated to Shanghai Jiao Tong University(China)	Human bone marrow	1 Melatonin-pretreated BMSC-Exos (MT-Exo); injected subcutaneously at least six sites per wound; dose not mentioned; at Day 0	PBS	Rats (Sprague-Dawley)	20 mm	1 Accelerated diabetic wound healing 2 Anti-inflammatory effect on macrophages by promoting M2 and inhibiting M1 polarization 3 Enhanced re-epithelialization (increased neoepithelium length) 4 Improved angiogenesis (α-SMA, CD31, Microfli perfusion) and collagen synthesis (Col I and III) 5 Activated the PTEN/AKT signaling pathway	PTEN/AKT signaling pathway
				2 BMSC-Exos; injected subcutaneously at least six sites per wound; dose not mentioned; at Day 0					
4	Pomatto *et al* [104], 2021	University of Turin(Italy)	Human bone marrow	BMSC-EVs + carboxymethylcellulose; applied on the wound; 1 × 10^9 in 25 μL of vehicle; at Day 0, 3, 7 and 10	carboxymethylcellulose high viscosity 10 mg/mL (25 μL)	Mice (NSG)	6 mm × 8 mm	Not effective and did not reduce the wound closure rate	–
			Human adipose tissue	AMSC-EVs + carboxymethylcellulose; applied on the wound; 1 × 10^9 in 25 μL of vehicle; at Day 0, 3, 7, 10 and 14				1 Accelerated cutaneous wound healing 2 Reduced size of the scar 3 Increased epithelial thickness and re-epithelization 4 Promoted angiogenesis (the number of vessels)	
5	Shi *et al*[139], 2020	Affiliated Hospital of Nantong university(China)	Human adipose tissue	1 mmu_circ_0000250-modified AMSC-Exos;injected subcutaneously at four sites around the wound;200 μg in 100 μL PBS;at Day 0	PBS (100 μL)	Mice (C57BL)	4 mm	1 Accelerated cutaneous wound healing 2 Reduced scar areas 3 Enhanced angiogenesis	mmu_circ_0000250/miR-128-3p/SIRT1-mediated autophagy

							(CD31, vessel density) 4 Suppressed apoptosis of skin tissue 5 Suppressed expression of miR-128-3p but promoted SIRT1 expression 6 Increased expression of autophagy-related gene (LC3)	
				2 AMSC-Exos; injected subcutaneously at four sites around the wound; 200 μg in 100 μL PBS; at Day 0				
6	Hu *et al*[138], 2021	Union Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology(China)	Rat bone marrow	1 Pioglitazone-treated BMSC-Exos (PGZ-Exos); injected subcutaneously(at least six sites per wound); 100 μg in 100 μL PBS; at Day 0	PBS (100 μL)	Rats (Sprague-Dawley)	15 mm	1 Accelerated cutaneous wound healing 2 Enhanced re-epithelization 3 Promoted collagen synthesis (Col I, Col III) and collagen deposition, indicating more superior ECM remodeling ability 4 Enhanced angiogenesis (VEGF, CD31) and blood flow of the wound	PTEN/PI3K/AKT/eNOS pathway
				2 BMSC-Exos; injected subcutaneously (at least six sites per wound); 100 μg in 100 μL PBS; at Day 0				
7	Yu *et al*[137], 2020	Shanghai Sixth People's Hospital affiliated to Shanghai Jiao Tong University; Second Military Medical University(China)	Human bone marrow	1 Atorvastatin-pretreated BMSC-Exos (ATV-Exos); injected subcutaneously (six points); dose not mentioned; at Day 0 2 BMSC-Exos; injected subcutaneously (six points); dose not mentioned; at Day 0	PBS	Rats (Sprague-Dawley)	20 mm	1 Accelerated cutaneous wound healing 2 Increased re-epithelization (more epithelial structures and longer neuroepithelium) 3 Promoted collagen synthesis and deposition, indicating more superior ECM remodeling ability (thicker wavy collagen fibers and more extensive collagen deposition arranged neatly) 4 Superior biosafety of the therapy of exosomes 5 Enhanced angiogenesis (CD31, α-SMA and Microfil perfusion)	miR-221-3p /PTEN/AKT/eNOS pathway

8	Zhao *et al*[123], 2021	Tongji University(China)	Human adipose tissue	1. AMSC-Exos; smeared at the wound; 200 μg in 200 μL PBS; 3 times/day, 2 wk 2 Recombinant human epidermal growth factor (rhEGF); smeared at the wound;3 times/day, 2 wk 3 AMSC-CM; smeared at the wound; 3 times/day, 2 wk	PBS;Untreated	Mice (db/db)	15 mm	1 Accelerated cutaneous wound healing 2 Exosomes entered the dermis of wounds after smearing 3 Mild hyperkeratosis and typical fibrous structures with new glands and hair follicles, implying enhanced tissue remodeling 4 Enhanced collagen synthesis (Col I, Col III), deposition and remodeling (large amounts, large area, regular arrangement and dense distribution of new collagen) 5 Enhanced cell proliferation and inhibited apoptosis 6 Increased blood vessel intensity and promoted angiogenesis (CD31, VEGF) 7 Repaired skin barrier functions (elevated expression levels Filaggrin, Loricrin, and AQP3) 8 Suppressed expression of inflammatory cytokines (IL-6, TNF-α, CD14, CD19 and CD68) 9 Negatively regulated MMP1 and MMP3 expression in promoting collagen synthesis	–
9	Tao *et al*[150], 2017	Shanghai Jiao Tong University Affiliated Sixth People's Hospital(China)	Human synovial membrane	1 miR-126-3p overexpressed SMSC-Exos + chitosan wound dressings; placed on the wound bed with pressure dressing; at Day 0 2 Chitosan wound dressings;	Untreated	Rats (Sprague-Dawley)	18 mm	1 Accelerated cutaneous wound healing 2 Enhanced angiogenesis (microcomputed tomography, CD31, α-SMA) 3 Promoted re-epithelial-	PI3K/AKT and MAPK/ERK signaling pathways

				placed on the wound bed with pressure dressing; at Day 0				ization, granulation tissue formation, collagen alignment and deposition, implying enhanced ECM remodeling 4 Accelerated development of hair follicles and sebaceous glands	
10	Ti *et al*[126], 2015	Chinese PLA General Hospital(China)	Human umbilical cord	1 LPS-pretreated HUCMSC-Exos; injected dispersively into the wound edge; 60 μg in 0.5 mL PBS; at Day 0 2 HUCMSC-Exos; injected dispersively into the wound edge; 60 μg in 0.5 mL PBS; at Day 0	Untreated	Rats	10 mm	1 Accelerated cutaneous wound healing 2 Decreased inflammatory cell infiltration 3 Regulate macrophage polarization to M2 macrophages 4 Promoted the appearance of new small capillaries	let-7b/TLR4/NF-κB/STAT3/AKT pathway
11	Li *et al*[136], 2020	The Fourth Affiliated Hospital of Harbin Medical University(China)	Mouse bone marrow	1 lncRNA H19 overexpressed BMSC-Exos; injected into the skin around the wound; at Day 0 2 BMSC-Exos; injected into the skin around the wound; at Day 0	Untreated	Mice (C57BL/6)	10 mm	1 Accelerated cutaneous wound healing. 2 Ameliorated inflammation of the wound (IL-10↑, IL-1β↓, TNF-α↓ and fewer inflammatory cells around the wound) 3 Promoted granulation tissue formation 4 Enhanced angiogenesis (Increased expression of VEGF, TGF-β1, α-SMA, and Col I) 5 Suppressed cell apoptosis 6 Interacted with miR-152-3p *via* PTEN-mediated PI3K/AKT signaling pathway (diminished miR-152-3p expression, elevated PTEN expression and decreased expression of PI3K, AKT and p-AKT)	lncRNA H19/miR-152-3p/PTEN/ PI3K/AKT signaling pathway
12	Shi *et al*.(2017) [142]	Chinese PLA General Hospital(China)	Human gingival tissue	1 GMSC-Exos+ chitosan/silk hydrogel sponge; covered the wound with restraining	1. PBS (100 μL);2. gauze (13 mm× 13 mm) covered the wound	Rats (Sprague-Dawley)	10 mm	1 Accelerated cutaneous wound healing	—

				bandage; 150 μg in 100 μl PBS; at Day 0, changed every 3 d 2 Chitosan/silk hydrogel sponge; covered the wound with restraining bandage; in 100 μL PBS; at Day 0, changed every 3 d				2 Promoted re-epithelialization, deposition and remodeling of ECM (more collagen deposition and thick wavy collagen fibers, the collagen fibers arranged in an orderly fashion similar to that of normal skin) 3 Enhanced angiogenesis (CD34, microvessel density) 4 Enhanced neuronal ingrowth (nerve fiber density)	
13	Xiao *et al*[151], 2021	Nan Fang Hospital of Southern Medical University(China)	Human adipose tissue	1 AMSC-Exos + human acellular amniotic membrane (hAAM) scaffold; covered on the wound; 100 μg in 100 μL PBS; at Day 0, every other day, 3 times in total 2 AMSC-Exos; covered on the wound;100 μg in 100 μL PBS; at Day 0, every other day, 3 times in total 3 hAAM patch; covered on the wound; at Day 0, every other day, 3 times in total	PBS (100 μL)	Mice (BALB/c)	10 mm	1 Accelerated cutaneous wound healing 2 Suppressed wound inflammatory responses (fewer inflammatory cells around the wound and higher recruitment of M2 macrophages to the wound sites) 3 Enhanced angiogenesis (CD31) 4 Enhanced extracellular matrix (ECM) deposition (Col III) 5 Promoted re-epithelialization (completed epithelial and dermal regenerated) 6 Failed regenerated hair follicle and sebaceous glands	–
14	Yan *et al*[152], 2022	Union Hospital, Tongji Medical College, Huazhong University of Science and Technology(China)	Human umbilical cord	1 HUCMSC-Exos injected locally to the wound site; 100 μL, 50 μg/ml; at days 0, 3, 5, 7, 9, and 11 2 HUCMSC-Exos injected locally to the wound site; 100 μL, 100 μg/mL; at days 0, 3, 5, 7, 9, and	PBS (100 μL)	Mice (C57BL/6J)	10 mm	1 Accelerated cutaneous wound healing 2 Reduced oxidative stress (ROS) 3 Promoted granulation tissue formation	–

				11				4 Enhanced angiogenesis (CD31, mean perfusion unit ratio)	
15	Geng *et al*[128], 2022	Jinzhou Medical University(China)	Rat bone marrow	1 BMSC-Exos + carboxyethyl chitosan-dialdehyde carboxy-methyl cellulose hydrogel; covered the wound; twice a day, two weeks 2 Carboxyethyl chitosan-dialdehyde carboxymethyl cellulose hydrogel; covered the wound; twice a day, two weeks	Untreated	Rats (Sprague-Dawley)	20 mm	1 Accelerated cutaneous wound healing 2 Promoted collagen deposition and remodeling, and fibrin regeneration 3 Enhanced antibacterial effects by significantly inhibiting bacterial growth 4 Skew macrophage functional polarity from M1 (iNOS) towards an anti-inflammatory M2 phenotype (CD206) 5 Decreased inflammatory factors (IL-1β, TNF-α) 6 Promoted proliferation of blood vessels and angiogenesis (CD31)	VEGF-mediated PI3K/AKT signaling pathways
16	Gondaliya *et al* [153], 2022	National Institute of Pharmaceutical Educationand Research(India)	Bone marrow	1 BMSC-Exos loaded with miR-155 inhibitor; injected subcutaneously; 0.1 μg/μL; 1 d after wound induction 2 BMSC-Exos; injected subcutaneously; 0.1 μg/μL; 1 d after wound induction 3 BMSC-Exos loaded with negative control sequences; injected subcutaneously; 0.1 μg/μL; 1 d after wound induction	Untreated	Mice (C57BL/6)	4 mm	1 Accelerated cutaneous wound healing 2 Declined miR-155 levels with a concomitant increase in FGF-7 3 Downregulated expression of MMP-2 and MMP-9 4 Declined expression of pro-inflammatory cytokines (TIMP-2, lymphotactin, sTNF RI, sTNF RII, and LIX); declined regulated upon activation, normal T cell expressed and secreted (RANTES) chemokine; downregulated pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and TGF-β1 5 Promoted re-epithelialization, collagen synthesis	–

								and deposition, angiogenesis (α-SMA) and vascularization (CAM)	
17	Dalirfardouei *et al*[125], 2019	Mashhad University of Medical Sciences(Iran)	Human menstrual blood	1 MenSC-Exos; injected intradermally; 10 μg in 100 μL of PBS; at Day 0 2 MenSCs; injected intradermally; 1 × 10^6 cells in 100 μL of PBS; at Day 0	PBS (100 μL)	Mice (C57BL/6)	8 mm	1 Accelerated cutaneous wound healing 2 Promoted re-epithelialization 3 Induced macrophage polarization from M1 (iNOS) to M2 (Arg) phenotype 4 Enhanced angiogenesis (VEGF, microvessel density) 5 Improved collagen deposition (upregulated Col I/Col III ratio at Day 7, downregulated at Day 14) 6 Decreased size of scar tissues 7 Decreased cellularity in the granulation tissue 8 Decreased *Rela* gene expression at Day 4, enhanced at Day 7.	NF-κB signaling pathway (possible)
18	Wang *et al*[124], 2022	Affiliated Hospital of Nantong University(China)	Rat bone marrow	1 BMSC-Exos + 50 mg/kg intraperitoneal tertbutylhydroquinone (tBHQ); injected subcutaneously of 4 sites at the base and edge of the wound; 100 μg/mL, 200 μL; at Day 0 and 7 2 BMSC-Exos + 200 μL intravenous Lenti-sh-NC; injected subcutaneously of 4 sites at the base and edge of the wound; 100 μg/mL, 200 μL; at Day 0 and 7 3 BMSC-Exos; injected subcutaneously of 4 sites at the base and edge of the wound; 100	PBS	Rats (Sprague-Dawley)	15 mm	1 Accelerated cutaneous wound healing 2 Promoted re-epithelialization and collagen deposition 3 Enhanced angiogenesis (CD31) 4 Reduced inflammation (decreased inflammatory cytokines TNF-α, IL-1β and increased anti-inflammatory cytokines IL-4, IL-10).	–

				μg/mL, 200 μL; at Day 0 and 7 4 BMSC-Exos + 200 μL intravenous Lenti-sh-Nrf2; injected subcutaneously of 4 sites at the base and edge of the wound; 100 μg/mL, 200 μL; at Day 0 and 7					
19	Sun *et al*[127], 2022	Nanjing Normal University; Nanjing University; Nanjing medical University; Nanjing Tech University(China)	Human umbilical vein	1 Engineering TNF-α/hypoxia-pretreated HUVMSC-Exos +PCOF; each subsequent day later, total 21 d 2 Engineering TNF-α/hypoxia-pretreated HUVMSC-Exos; each subsequent day later, total 21 d 3 Vancomycin; each subsequent day later, total 21 d 4 PCOF; each subsequent day later, total 21 d	PBS		Mice (C57BL/6)	15 mm (*S.aureus*-infected chronic wounds)	1 Accelerated cutaneous wound healing 2 Reduced bacterial burden and suppressed bacterial colonization in the wound sites 3 Reduced the inflammatory response (immune cells counting); decreased proinflammatory cytokines (TNF-α, IL-1β, IL-6); induced M2 (CD206) macrophages polarization 4 Promoted collagen deposition and remodeling, granulation formation, re-epithelialization and enhanced proliferation of fibroblasts 5 Enhanced cell proliferation (Ki67) 6 Suppressed oxidative stress induced by bacteria and peroxide substrates (reduced the content of oxidative biomarkers and (MDA) increased the antioxidant mediators (GSH-Px, SOD) 7 Promoted angiogenesis (upregulated miR-126, HIF-1α, VEGF, CD31 and α-SMA; increased neovascularization) 8 *In vivo* biosafety (blood system, heart, liver, kidney and other organs)	miR-126/SPRED1/RAS/ERK pathway (possible)

20	Li *et al*[147], 2016	Shanghai Normal University; Shanghai Jiao Tong University Affiliated Sixth People's Hospital(China)	Human synovial tissue	1 miR-126-3p overexpressed SMSC-Exos + hydroxyapatite/chitosan composite hydrogel; placed on the wound bed with pressure dressing 2 Hydroxyapatite/chitosan composite hydrogel; placed on the wound bed with pressure dressing	Untreated	Rats (Sprague-Dawley)	18 mm	1 Accelerated cutaneous wound healing 2 Enhanced angiogenesis (μCT), formation and maturation of new vessels (CD31, α-SMA) 3 Promoted re-epithelialization, granulation tissue maturation, collagen alignment and deposition that indicated improved ECM remodeling 4 Accelerated growth of follicles and sebaceous glands	Activated MAPK/ERK and PI3K/AKT pathways
21	Zhang *et al*[148], 2021	Jinzhou Medical University(China)	Human umbilical cord	1 HUCMSC-Exos + polyvinyl alcohol (PVA)/alginate (Alg) nanohydrogel; locally transplanted; 300 μL; once a day 2 HUCMSC-Exos; locally transplanted; 300 μL; once a day 3 PVA/Alg nanohydrogel; locally transplanted; 300 μL; once a day	Untreated	Rats (Sprague-Dawley)	15 mm × 2 mm	1 Accelerated cutaneous wound healing 2 Enhanced re-epithelialization and hair follicles formation 3 Promoted collagen deposition and remodeling (increased and orderly arranged collagen fibers) 4 Promoted angiogenesis (CD31, α-SMA, SR-B1, VEGF)	ERK1/2 pathway
22	Han *et al*[154], 2022	The First Affiliated Hospital of Zhengzhou University(China)	Human bone marrow	1 lncRNA KLF3-AS1 overexpressed BMSC-Exos; injected *via* tail vein; 100 μL; at Day 0 2 Negative control silenced BMSC-Exos;injected *via* tail vein;100 μL;at Day 0 3 Negative control overexpressed BMSC-Exos; injected *via* tail vein; 100 μL; at Day 0 4 lncRNA KLF3-AS1 silenced BMSC-Exos; injected *via* tail vein; 100 μL; at Day 0	Untreated	Mice (BALB/c)	Not mentioned	1 Accelerated cutaneous wound healing 2 Minimized weight loss. 3 Reduced inflammation (decreased IL-6 and IL-1β) 4 Promoted angiogenesis (CD31), collagen deposition and follicle regeneration 5 Decreased expression of miR-383 and increased VEGFA	lncRNA KLF3-AS1/miR-383/VEGFA signaling pathway
23	Ding *et al*[155], 2019	Shanghai Jiao Tong University Affiliated	Human bone marrow	1 Deferoxamine-preconditioned BMSC-Exos (DFO-Exos); injected	PBS (100 μL)	Rats (Sprague-Dawley)	20 mm × 2 mm	1 Accelerated cutaneous wound healing	miR-126/PTEN/PI3K/AKT pathway

		Sixth People's Hospital(China)		subcutaneously around the wounds at four sites; 100 μg in 100 μL PBS; at Day 0 2 BMSC-Exos; injected subcutaneously around the wounds at four sites; 100μg in 100μL PBS; at Day 0				2 Enhanced re-epithelialization and lower scar formation 3 Promoted collagen deposition (increased wavy collagen fibers) 4 Promoted angiogenesis (vessel density by micro-CT, CD31, α-SMA)	
24	Bian *et al*[135], 2020	Chinese PLA General Hospital(China)	Human decidua	dMSC-sEVs; injected around the wounds at 4 sites (25 μL per site); 100 μL, 5.22×10^{11} particles/mL; at Day 7, 14, 21and 28	PBS (100 μL)	Mice (BKS-db)	16 mm	1 Accelerated cutaneous wound healing 2 Reduced scar width 3 Accelerated collagen deposition (larger and better-organized collagen deposition) 4 Enhanced fibroblast proliferation (PCNA), migration (CXCR4), and differentiation abilities of fibroblast 5 Promoted angiogenesis (α-SMA) 6 Improved fibroblast senescent state (p21)	RAGE/RAS; Smad pathways
25	Zhang *et al*[156], 2022	Xijing Hospital of Fourth Military Medical University(China)	Human adipose tissue	AMSC-Exos; injected subcutaneously; 200 μg; 3 d after wound induction, for three consecutive days	PBS (100 μL)	Mice (db/db)	10 mm	1 Accelerated cutaneous wound healing 2 Enhanced re-epithelialization 3 Promoted angiogenesis (CD34, VEGF) 4 Improved oxidative stress (MDA, T-AOC, SOD) 5 Reduced inflammatory cytokines (IL-1β, IL-6, TNF-α, MCP-1)	SIRT3/SOD2 pathway
26	Born *et al*[157], 2021	University of Maryland; Johns Hopkins University School of Medicine(USA)	Human bone marrow	1 HOX transcript antisense RNA (HOTAIR) overexpressed BMSC-EVs; injected around the wound in a cross pattern of four	PBS (50 μL)	Mice (db/db)	8 mm	1 Accelerated cutaneous wound healing 2 Promoted angiogenesis	–

				sites; 50 μg in 50 μL PBS; at Day 3, four times 2 BMSC-EVs; injected around the wound in a cross pattern of four sites; 50 μg in 50 μL PBS; at Day 3, four times				(CD31, VEGFA)	
27	Teng *et al*[158], 2022	Jiangnan University (China)	Human umbilical cord	HUCMSC-Exos; injected subcutaneously around the wounds at four sites; 100 μL (100 μg/mL); at Day 0	PBS (100 μL)	Rats (Sprague-Dawley)	10 mm	1 Accelerated cutaneous wound healing 2 Inhibited chronic inflammation: (decreased number of inflammatory cells); inhibited pro-inflammatory cytokines (TNF-α); induced M2 (CD206) macrophages polarization 3 Enhanced re-epithelialization 4 Promoted angiogenesis (increased new blood vessels, CD31, VEGF) 5 Promoted collagen synthesis and skin regeneration	–

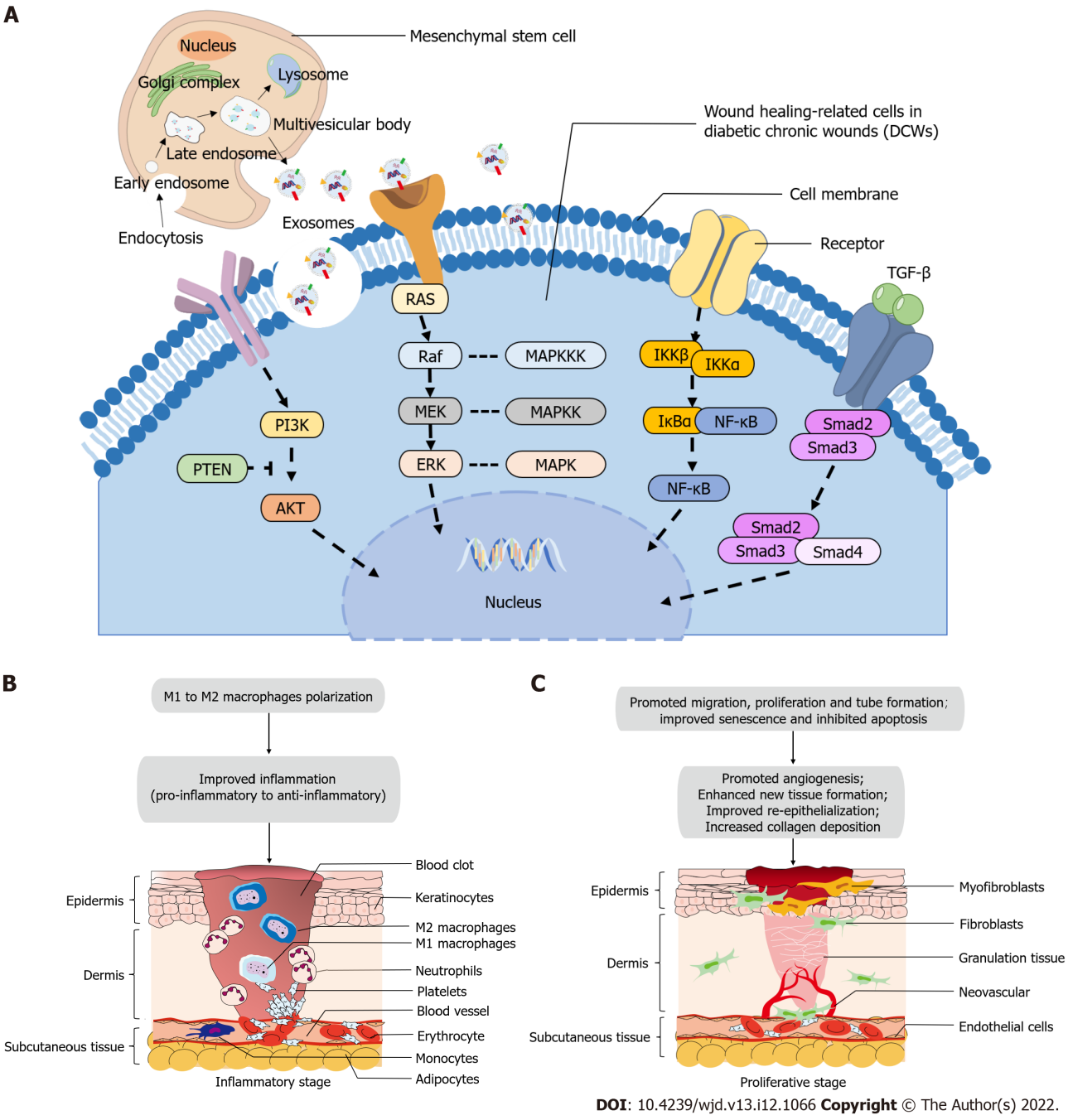
HUCMSC-Exos: Human umbilical cord mesenchymal stem cell derived exosomes; PF-127: Pluronic F-127; PBS: Phosphate buffered saline; MVD: Microvascular density; Ki67: Nucleus related antigen; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor; F127: Pluronic F127; OHA: Oxidative hyaluronic acid; EPL: Poly-ε-L-lysine; Col I: Collagen I; Col III: Collagen III; α-SMA: Alpha smooth muscle actin; BMSC-Exos: Bone marrow mesenchymal stem cell derived exosomes; PTEN: Phosphatase and tensin homolog; BMSC-EVs: Bone marrow mesenchymal stem cell derived extracellular vesicles; AMSC-EVs: Adipose tissue mesenchymal stem cell derived extracellular vesicles; AMSC-Exos: Adipose tissue mesenchymal stem cell derived exosomes; SIRT1: Silent mating type information regulation 2 homolog-1; LG3: Light chain 3; ECM: Extracellular matrix; PI3K: Phophatidylinositol3-kinase; eNOS: Endothelial nitric oxide synthase; AMSC-CM: Adipose tissue stem cell conditioned medium; AQP3: Recombinant aquaporin 3; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor alpha; SMSC-Exos: Synovial membrane mesenchymal stem cell derived exosomes; MAPK: Mitogen-activated protein; ERK: Extracellular signal regulated kinase; let-7b: MicroRNA let-7b; TLR4: Toll like receptor 4; NF-κB: Nuclear factor kappa-B; STAT3: Signal transducer and activator of transcription 3; Il-10: Interleukin 10; IL-1β: Interleukin 1β; GMSC-Exos: Gingival tissue mesenchymal stem cell derived exosomes; iNOS: Inducible nitric oxide synthase; sTNF RI: Soluble tumor necrosis factor receptor I; sTNF RII: Soluble tumor necrosis factor receptor II; FGF-7: Fibroblast growth factor 7; LIX: Lipopolysaccharide-induced CXC chemokine; CAM: Chick chorioallantois membrane; MenSC-Exos: Menstrual blood mesenchymal stem cell derived exosomes; MenSCs: Menstrual blood-derived mesenchymal stem cells; Arg: Arginase; Lenti-sh-Nrf2: Lentiviral shRNA targeting Nrf2; Lenti-sh-NC: Lentiviral control shRNA; HUVMSC-Exos: Human umbilical vein mesenchymal stem cell derived exosomes; PCOF: Polydopamine modified reductive covalent organic frameworks; *S.aureus*: *Staphylococcus aureus*; MDA: Malondialdehyde; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; SR-B1: Scavenger receptor class B type I; dMSC-sEVs: Decidua mesenchymal stem cell derived extracellular vesicles; PCNA: Proliferating cell nuclear antigen; CXCR4: CXC-chemokine receptor 4; p21: Cyclin-dependent kinase inhibitor 1A; RAGE: Receptor for advanced glycation end products; RAS: rat sarcoma; T-AOC: Total antioxidant capacity; ,MCP-1: Monocyte chemoattractant protein-1; SIRT3: Silent mating type information regulation 2 homolog 3.

Thus, efficient, stable, safe, and mass-producible stem cells and related products for the treatment of diabetic wounds are yet to be explored and developed. More research is required in future clinical trials and routine practice to determine the most effective cell sources for diabetic wounds; to establish optimal large-scale culture conditions of MSCs; to solve the preparation problem of huge heterogeneity of exosome components; to explore standardized isolation, quality control, purification, and characterization techniques of MSC-Exos; and to determine the best approach for long-term storage[162].

Table 2 Clinical trials of exosomes in treating various wounds

Start year	Institution (Nation)	Type of wounds	Intervention	Autologous/Allogeneic	Administration, frequency	Patients number	Follow-up period	Outcome measures	Phase	Study design	ClinicalTrials.gov identifier	Status
2022	Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University (China)	Full-layer skin wounds	Adipose tissue derived exosomes(200-300 mL of the subject adipose tissue)	Autologous	Applied directly to the wound (mixed with sterile hydrogel), twice a week	5	4 wk	**Primary:** Percentage of wound healing	Not Applicable	Non-randomized, single group assignment, open label	NCT05475418	Not yet recruiting
2015	Kumamoto University (Japan)	Intractable cutaneous ulcers (*e.g.*, rheumatic disease, peripheral arterial disease, chronic venous insufficiency, decubitus or burns)	Plasma-derived exosomes (Plasma samples will be filtered through 0.45 μm and 0.20 μm filters. The samples will be filtered through 0.02 μm filter to trap exosomes with the filter. Saline solution will be loaded from the other side of the 0.02 μm filter to obtain exosome rich buffer.)	Autologous	Applied to the ulcer, daily	5	28 d	**Primary:** Ulcer size (length, width, depth) **Secondary:** Pain of cutaneous wounds (VAS)	Early Phase 1	Non-randomized, single group assignment, open label	NCT02565264	Unknown
2023	Aegle Therapeutics (USA)	Dystrophic Epidermolysis Bullosa (DEB); chronic wounds (< 20% closure of wound during observation period); 10-50 cm^2	Bone marrow mesenchymal stem cells derived extracellular vesicle (AGLE-102)	Allogeneic	Multiple administrations of 2 ascending dose levels of AGLE-102; (up to 6 administrations); (each administration will occur 14 ± 7 d but no less than 7 d apart); (each administration no more than 3 mo); (wound closes prior to 6 administrations, no additional doses will be given)	10	8 mo; if the wound closes before receiving all 6 doses, for 4 mo after the wound closes	**Primary:** Dose limiting toxicity **Secondary:** Wound size	Phase 1/2	Non-randomized, multicenter, ascending dose, single group assignment, open label	NCT04173650	Not yet recruiting
2019	Mayapada Hospital (Indonesia)	Chronic wounds	Human Wharton's Jelly mesenchymal stem cells conditioned medium (WJ-MSC-CM)	Allogeneic	Applied to the wound (the conditioned medium gel), every week	38	2 wk	**Primary:** Success rate of chronic ulcer healing	Phase 1	Non-randomized, single group assignment, open label	NCT04134676	Completed

Researchers also need to fully understand the abilities, loss, distribution, diffusion efficiency, and clearance efficiency of exosomes after transporting them to target areas. Physical, chemical, or biological methods for preconditioning, genetic engineering, and transfection are used to specifically enhance a certain therapeutic potential to achieve relatively better wound healing than native exosomes, thus becoming new treatment directions[163]. Additionally, combining exosomes with biomaterials is



DOI: 10.4239/wjd.v13.i12.1066

Figure 1 Molecular mechanism of mesenchymal stem cell-derived exosomes in diabetic cutaneous wound healing. A: signaling pathways most frequently studied in diabetic wound models and may potentially confirmed in diabetic chronic wounds; B: microenvironmental changes in inflammatory stage of wound healing after using mesenchymal stem cell-derived exosomes; C: microenvironmental changes in proliferative stage of wound healing after using mesenchymal stem cell-derived exosomes. PTEN: Phosphatase and tensin homolog; PI3K: Phophatidylinositol3-kinase; Akt/PKB: Protein kinase B; RAS: Rat sarcoma; Raf: Rapidly accelerated fibrosarcoma; MAPK: Mitogen-activated protein; ERK: Extracellular signal regulated kinase; NF-κB: Nuclear factor kappa-B; TGF-β: Transforming growth factor-β; Smad2/3/4: Drosophila mothers against decapentaplegic.

possible to create bioactive dressings to enhance or combine repair ability, provide local microenvironment stability, and achieve sustained release of exosomes[74]. Additionally, starting clinical trials as soon as possible is necessary to verify the optimal dosages, administration methods, and efficacy evaluation of MSC-Exos in clinical patients, looking forward to its broad application prospects in promoting DCW healing in clinical practice[162].

CONCLUSION

DCWs, which are one of the most common chronic refractory wounds, pose a heavy burden to patients, families, and society. Current studies have suggested that MSC-Exos can play an important role in

various aspects of wound healing and hold sufficient promise for promoting diabetic wound healing. However, recent clinical applications of MSC-Exos in DCW repair are still limited. Moreover, clinical translational issues, such as exosome production, isolation, purification, and storage processes, the most effective route of administration and dose, and efficacy evaluation remain. Accurate and efficient exosome products need to be established, and experiments in animals that have a greater resemblance to human skin tissues and clinical trials need to be initiated as soon as possible to validate the optimal dosage and administration, and efficacy evaluation for using MSC-Exos to provide safety assurance for further clinical applications. Modification of MSC-Exos and integration with biomaterials to improve their efficacy and reduce their elimination rate may be a promising direction. We look forward to the clinical application of MSC-Exos for diabetic wound healing.

FOOTNOTES

Author contributions: Ran XW and Chen LH designed the research study; Wu J, Sun SY and Li Y performed the literature retrieval; Wu J and Chen LH wrote the manuscript; Ran XW reviewed and revised the manuscript; All authors have read and approved the final manuscript.

Supported by the 1.3.5 Project for Disciplines of Excellence, West China Hospital, Sichuan University, No. ZYGD18025.

Conflict-of-interest statement: Authors declare no conflict of interests associated with this manuscript.

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S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1096-1105

DOI: 10.4239/wjd.v13.i12.1096

ISSN 1948-9358 (online)

MINIREVIEWS

Prehabilitation of overweight and obese patients with dysglycemia awaiting bariatric surgery: Predicting the success of obesity treatment

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Estabile P, Brazil; Obando A, Nicaragua; Salimi M, Iran

Received: July 21, 2022
Peer-review started: July 21, 2022
First decision: September 4, 2022
Revised: September 9, 2022
Accepted: November 2, 2022
Article in press: November 2, 2022
Published online: December 15, 2022

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Abstract

Bariatric surgery offers the best health results in overweight and obese patients but is not a risk and/or complication-free treatment. In cases with additional hyperglycemia, the burden of surgery can be even higher and alter both short-term and long-term outcomes. Although bariatric surgery offers glycemic improvements and in the case of early onset diabetes disease remission, weight loss results are lower than for obese patients without diabetes. Different multimodal programs, usually including interventions related to patients' performance, nutritional and psychological status as well as currently available pharmacotherapy before the surgery itself might considerably improve the immediate and late postoperative course. However, there are still no clear guidelines addressing the prehabilitation of obese patients with dysglycemia undergoing bariatric surgery and therefore no unique protocols to improve patients' health. In this minireview, we summarize the current knowledge on prehabilitation before bariatric surgery procedures in patients with obesity and dysglycemia.

Key Words: Bariatric surgery; Obesity; Dysgylcemia; Diabetes outcome; Prehabilitation

Core Tip: The prehabilitation of bariatric surgery patients is an insufficiently investigated area of research. Adequate perioperative preparation for patients awaiting bariatric surgery could present one of the main determinants of predicting the success of surgical treatment, especially in patients with associated dysglycemia. A combination of calorie restrictive diet, structured exercise program, psychological support, and anti-obesity pharmacotherapy should be implemented in the perioperative care of candidates for bariatric procedures. This multimodal approach has the most promising potential to promote 5% weight loss at least thus affecting chronic inflammation and insulin resistance, the main culprits of bariatric surgery resistance.

Citation: Cigrovski Berkovic M, Bilic-Curcic I, Mrzljak A, Canecki Varzic S, Cigrovski V. Prehabilitation of overweight and obese patients with dysglycemia awaiting bariatric surgery: Predicting the success of obesity treatment. *World J Diabetes* 2022; 13(12): 1096-1105
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1096.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1096

INTRODUCTION

Obesity is a chronic debilitating disease with many health-related consequences. Nearly 39% of the worldwide adult population in 2019 met the criteria of being overweight and obese, and had multiple comorbidities[1,2]. In the case of additional derangements in glucose metabolism, such as glucose intolerance or diabetes whose incidence increases with increasing body mass index (BMI), patients have an even worse long-term prognosis, with accentuated cardiovascular risk, morbidity, and mortality[3].

Even the accumulation of free fat mass in the legs, arm, and trunk area is reversely associated with diabetes as was demonstrated in a recent study[4]. Moreover, when weight reduction results (due to lifestyle interventions, pharmacotherapy, or metabolic surgery) are compared to obese patients with and without diabetes, later are always more humble, suggesting the necessity for a structured and multimodal approach[5].

Weight management aimed at weight reduction has favorable metabolic, and mental health benefits in obese patients. A healthy lifestyle, including physical activity, is one of the pillars of weight management, impacting overall cardiometabolic health and well-being[6]. In addition, newly available anti-obesity drugs can lead to potent weight loss results, but the most powerful strategy includes bariatric surgery. Different surgical approaches can be selected, some with malabsorptive effects and others, such as gastric sleeve-resection do not have malabsorptive effects.

Malabsorptive procedures lead to nutritional risks, which might also exist preoperatively, regardless of patients' BMI. Therefore, preoperative nutritional status assessment and cardiorespiratory fitness status might be important parameters in decision making, treatment planning, and psychiatric evaluation. The Enhanced Recovery after Bariatric Surgery protocol suggests that a higher preoperative fitness level leads to improved outcomes and fewer postoperative complications[7]. Unfortunately, current medical care does not routinely include a physical exercise component for bariatric surgery patients. Moreover, < 10% of bariatric surgery patients meet the current physical activity recommendations, although it has been shown that two weeks before surgery, 40% of obese patients would feel ready to start exercise[8].

In addition, prehabilitation might be the key to improving responsiveness to metabolic surgery, especially in patients with dysglycemia, one of the common comorbidities in overweight/obese patients that must be addressed preoperatively[9].

In this minireview, we will focus on multimodal prehabilitation of patients undergoing bariatric surgery and specifically look into data on patients with coexisting dysglycemia.

ROLE OF EXERCISE

Exercise is a cornerstone of a healthy lifestyle and disease prevention, and sedentarism, lack of exercise, or nonattainment of physical exercise goals have been strongly correlated with chronic non-communicable diseases such as obesity, metabolic syndrome, and type 2 diabetes mellitus (T2DM)[10,11]. The inclusion of physical exercise in multimodal preconditioning programs for patients undergoing

different surgical procedures has been in the research scope of numerous investigations[12].

The role of exercise programs before and after bariatric surgery procedures might be important both from the aspect of reduction of perioperative and postoperative complications and as a means of retaining weight loss results achieved by surgery and acquisition of a healthy lifestyle[13,14]. Unfortunately, despite convincing beneficial outcomes reported from other surgical procedures, structured perioperative exercise programs are barely/rarely used perioperatively for bariatric procedures. According to the literature, physical exercise can contribute to approximately 4% excess weight loss, and when exercise is performed post-bariatric surgery, it results in an additional 3.6 kg weight loss[15]. The beneficial effects of exercise on anthropometric measures (weight loss, reduction of fat mass, and reduction of neck circumference) accompanied by improvement in physical performance (measured by the 6-min walk test) and quality of life are well documented[16,17]. There are, however, no clear recommendations on validated programs concerning starting the exercise before bariatric surgery, type of exercise, the intensity of exercises, duration of exercise sessions, or the comparison of different exercise types concerning short-term and long-term outcomes. Moreover, the literature is mainly focused on exercise performed post-bariatric surgery procedures and how it might help retain weight loss and cut cardiovascular risk compared to preoperative exercise programs[18,19].

A few studies that have assessed the value of preoperative exercise suggest benefits in fitness level and achievement of presurgery weight loss. Specifically, a 12-wk pre-bariatric surgery program including endurance and resistance exercises suggests improvements in fitness and quality of life-extending one year post-operatively[14,16,20]. In addition, studies using endurance and resistance training as a pre-bariatric surgery intervention reported improvements in weight and functional capacity, comorbidities, and quality of life[21,22].

Recently published data from a randomized controlled trial, although having major adherence issues, suggested the benefit of resistance exercises with elastic bands involving large muscle groups of the upper and lower extremities in the perioperative period of obese patients awaiting bariatric surgery together with respiratory prehabilitation[23].

Obese patients with dysglycemia (prediabetes or diabetes) are at higher risk of diabetes and obesity-related comorbidities[24].

In the study by Hickey *et al*[25] a seven-day 60-min daily exercise program led to a significant decrease in fasting plasma insulin level, suggesting improvements in tissue insulin sensitivity, which is particularly important for overweight/obese patients with dysglycemia. During 24 wk of low-intensity endurance training, in addition to anthropometric parameter measurements, Marcon *et al*[26] found substantial improvements in systolic and diastolic blood pressure, lipid and glucose levels, and patients' performance. A study by Woodlief *et al*[27] focusing on exercise dose after Roux-en-Y gastric bypass surgery showed that even a modest amount of structured exercise leads to improvements in insulin sensitivity but that higher volumes of exercise are needed for more profound health benefits.

On the other hand, Gilbertson *et al*[28] investigated the effects of aerobic exercise (30 min/d, 5 d/wk, at home, walking at the intensity of 65%-85% peak heart rate during 30 d) on metabolic and short-term postoperative outcomes of bariatric patients. They found a significant decrease in calorie intake, increase in VO_2peak, decrease in high sensitivity C-reactive protein (hsCRP), cytokeratin 18 and improvement in quality of life, decreased sugar intake, improved whole-body insulin sensitivity, and glucose levels together with a shorter hospital stay in patients who were in the exercise group[28]. Moreover, from the aspect of choosing a better exercise type, interval training might be superior to moderate-intensity continuous training in terms of reducing fat mass[29].

The main problem in objectively assessing the contribution of exercise programs on weight loss outcomes, besides the lack of randomized controlled trials, is the lack of structured exercise, poor patient adherence, and the self-reported measurement of exercise limiting interpretation of the results.

ROLE OF DIET

Restrictive calorie intake is widely advocated for obese patients undergoing metabolic surgery, and a weight loss of 5%-10% is generally mandatory before patients are considered as candidates for bariatric surgery, primarily as a means of assessing patient's motivation and adherence to follow-up after the surgery[30].

Currently, different dietary interventions mainly investigated in a non-randomized and uncontrolled manner, such as a low-calorie diet (800/1200 kcal daily) or a very-low-calorie diet (600 kcal per day), were shown to reduce weight preoperatively (4.2% and 5.8%, respectively) with no difference in inducing a reduction in liver volume and having similar effects on surgical complications, length of hospital stay and biochemical parameters[31]. In addition, very low-calorie ketogenic diets have recently been investigated in the context of weight reduction in obese patients. Although concern is raised due to their ability to induce catabolism, enhance oxidative stress response, and through high protein intake, induce a negative metabolic response, data available from a few non-randomized studies suggest that the mentioned dietary regimen when used 30 d before bariatric surgery and in a sequential way with low calorie and a very low-calorie diet adds beneficial effects in terms of better weight

reduction, waist circumference, visceral fat reduction, and improvement in glycemic and lipid profiles accompanied by a mean 30% reduction in liver volume[32-34].

It is still unclear whether overweight and obese patients benefit from short-term dietary weight loss interventions while changes in the level of circulating mediators of appetite such as leptin, ghrelin, and GIP might favor long-term weight regain[35]. Moreover, overweight/obese patients might also be at nutritive risk, which might escalate if restrictive diets are not controlled[36]. Numerous studies reported multiple micronutrient deficiencies in obese patients[37-39], while Schiavo *et al*[40] showed that preoperative micronutrient supplementation leads to the prevention of micronutrient deficit in the postoperative period. Therefore, current guidelines support the preoperative nutritional status screening of all patients awaiting bariatric surgery[41].

A meta-analysis including 6060 patients showed significant weight reduction achieved through preoperative dietary restriction led to significant weight loss and 27% shorter duration of hospital stay, but with no difference regarding perioperative morbidity and mortality[42]. Stefura *et al*[43] prospectively collected data from 909 bariatric patients treated by ERAS principles and depicted predictors of success in losing > 5% of initial weight as positive (diabetes mellitus, obstructive sleep apnea, and previous surgery) or negative (steatohepatitis, respiratory disorders). Although there was no influence of preoperative weight loss on perioperative morbidity or mortality, patients who lost > 5% in the perioperative period had better weight loss results post-surgery[43].

The efficacy of calorie restriction (very-low-calorie diet and more recently very low-calorie ketogenic diet) in weight loss potential is an interesting bridging therapy before bariatric surgery but is still under debate due to the lack of large randomized studies addressing the issues around the effect on postoperative complications.

ROLE OF PHARMACOTHERAPY IN PREHABILITATION

A certain number of individuals are resistant to the weight loss effects of bariatric surgery due to multiple reasons such as the level of chronic inflammation, presence of T2DM, age, gender, and ethnicity[44].

Chronic inflammation and increased circulating levels of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α caused by white visceral adipose tissue could be one of the main reasons for bariatric surgery resistance independent of all other factors[45]. In responsive individuals, bariatric surgery reduces pro-inflammatory cytokines promoted by weight loss and attenuates insulin resistance[46-48]. Therefore, reducing pre-operative inflammation could improve response to bariatric surgery[49].

To date, several studies have demonstrated that severe dysglycemia, duration of diabetes, and anti-hyperglycemic therapy at the time of surgical procedure are the key factors in predicting response to bariatric surgery[50-54]. Whether hyperglycemia or insulin resistance are the main culprits in bariatric surgery resistance remains to be seen but improving glycemic regulation and insulin sensitivity could be the most important pre-operative pharmacological targets to improve responsiveness to bariatric surgery.

In addition, unchangeable factors, including aging, female sex[55,56], and Hispanic and African American races[57], are associated with higher rates of bariatric surgery failure. Therefore, influencing modifiable risk factors seems to be the most reasonable approach to improve the success of bariatric procedures.

Although lifestyle modifications such as physical activity and diet play a major role in the prehabilitation of bariatric patients, adherence to lifestyle changes remains an elusive and poorly attainable goal [42]. Implementing pharmacological options that reduce insulin resistance and chronic inflammation by lowering body weight preoperatively in patients with or without diabetes has great potential to improve the response to bariatric surgery.

There are several weight loss agents available on the market. One of the most frequently used is liraglutide, a long-acting glucagon-like peptide 1 receptor agonist (GLP 1 RA) approved for the treatment of T2DM and obesity due to its mechanism of action based on delayed gastric emptying, central reduction of appetite, and stimulation of glucose-dependent insulin secretion[58,59]. The efficacy and safety of liraglutide 3 mg daily were assessed in the phase III clinical trial program SCALE, demonstrating greater improvement compared to placebo with regard to HbA1c, blood pressure, lipid reduction, and health-related quality of life in overweight people and obese patients[58-61]. However, most research seems to focus on the role of liraglutide in post-operative management, preventing weight regain, and promoting further weight loss. At the same time, data on perioperative administration are scarce. The effectiveness of liraglutide in the prehabilitation of bariatric patients was demonstrated for the first time in a retrospective cohort analysis by Wood *et al*[62] in which therapy with GLP-1 receptor agonists in combination with other anti-diabetic medication prior to bariatric surgery led to higher T2DM remission rates, short- and long-term, compared to therapy with other anti-diabetic medications alone[62,63]. Recently, a case series also demonstrated the potential benefit of short-term therapy with liraglutide prior to bariatric surgery[64]; however, data from randomized

clinical trials (RCTs) are lacking.

Presently, there are several retrospective studies demonstrating the efficacy of liraglutide therapy in patients that underwent bariatric surgery with inadequate weight loss or weight regain[65,66], including one RCT investigating liraglutide effects compared to placebo on total weight loss and excess body weight loss added early after laparoscopic sleeve gastrectomy in obese individuals[63]. Liraglutide significantly improved the resolution of dysglycemia and weight loss effects of the surgical procedure compared to placebo.

Another promising agent from the same class is semaglutide, a long-acting GLP 1RA with proven effects on diabetes management and weight loss and recently approved by the FDA for both indications.

Semaglutide has improved pharmacokinetic properties compared to liraglutide, enabling once-weekly administration and greater efficacy[67]. In a phase III clinical trial assessing the efficacy and safety of semaglutide 2.4 mg in obesity treatment, greater reductions in body weight were observed after 68 wk with once-weekly semaglutide 2.4 mg sc *vs* placebo (mean change from baseline -14.9% *vs* -2.4%; ETD -12.4%; 95%CI: -13.4 to -11.5; $P < 0.001$)[68-71]. Similar results were found in a 68-wk phase III study (STEP 3) comparing the effects of semaglutide 2.4 mg *vs* placebo in overweight or obese adults without diabetes. The mean body weight decreased 16% with semaglutide, compared to 5.7% with placebo ($P = 0.0001$)[70]. No data are available on semaglutide in the prehabilitation of bariatric patients.

Tirzepatide belongs to an emerging new class of drugs called twincretins, dual receptor agonists of the glucose-dependent insulinotropic polypeptide (GIP) and GLP-1[72]. In the phase III clinical trial program SURPASS, designed to assess the efficacy and safety of tirzepatide 5, 10, and 15 mg as a treatment to improve glycemic control in patients with T2DM, tirzepatide demonstrated impressive results in terms of glycemic regulation and weight management[73,74]. In SURPASS-2, a higher dose of tirzepatide (15 mg) had more pronounced weight loss effects compared to semaglutide 1 mg (13.1% *vs* 6.7%) as well as better anti-hyperglycemic effects (2.3% *vs* 1.86%)[74].

Older anti-obesity medications such as orlistat, phentermine/topiramate, and naltrexone/bupropion have low efficacy and cause a drop in body weight up to 3%-7% compared to placebo with unfavorable safety profiles[75]. Liraglutide also induces similar weight loss but with a more acceptable safety profile. Consequently, the efficacy of semaglutide 2.4 mg and tirzepatide 15 mg in terms of weight loss effects is extremely significant, highlighted by the fact that approximately 75% of patients treated with semaglutide 2.4 mg or tirzepatide 15 mg experience 10% to 15% body weight loss accompanied by well-known side-effects such as nausea, vomiting, diarrhea and obstipation[76].

Therefore, these new agents could represent a new era in optimizing the medical care of bariatric surgery patients with the potential to significantly influence surgery outcomes. Further prospective randomized trials are necessary to determine the significance of these new classes of anti-obesity medications in the prehabilitation of bariatric surgery patients.

ROLE OF PSYCHOLOGICAL SUPPORT

Numerous studies have demonstrated a link between obesity and psychological disorders in patients awaiting bariatric surgery, the most common being anxiety, depression and binge eating disorders (BED)[77-79]. However, the effect of psychological status perioperatively on the success of bariatric surgery remains to be clarified due to large heterogeneity within the same psychiatric diagnosis influencing eating patterns. For instance, in a recently published study, better weight loss was associated with depression and BED diagnosis[80] as opposed to other findings linking higher levels of psychopathology with the diminished success of weight reduction[81,82]. Moreover, the results of the latest meta-analysis including published studies on psychological interventions in patients undergoing bariatric surgery were ambiguous regarding the usefulness of psychological support on bariatric surgery outcomes[83]. Therefore, further research on this topic is needed to assess if the benefit of psychological therapy really exists.

FUTURE IMPLICATIONS

Without a doubt, lifestyle modifications based on implementing structured exercise programs and nutritional plans offer great benefits in the prehabilitation of patients awaiting bariatric surgery, especially those with associated dysglycemia. The ultimate goal is achieving a minimum 5% weight loss and improving cardiorespiratory fitness and increasing basal rate consequently promoting further postoperative weight loss and bariatric surgery responsiveness as well as reducing postoperative complications and mortality. However, clear recommendations regarding the most efficient exercise protocols and calorie-restrictive diets are lacking and further prospective studies are needed to establish effective and safe protocols to upgrade peri and postoperative care as well as the short- and long-term outcomes of surgery. One should not forget the influence of patient characteristics, psychological profile, social conditions, and behavioral responses to the operation, which also have a great impact on surgery success requiring the development of protocols for psychological support. Furthermore, current

Table 1 Proposed recommendations for the perioperative care of all bariatric surgery patients, especially those with associated dysglycemia

Prehabilitation- treatment modality	Potential advantages and clinical rationale
Exercise	
Resistance and endurance training	Short- and long-term improvements in weight and functional capacity, comorbidities, quality of life, improvements in tissue insulin sensitivity
Aerobic training	Short-term decrease in calorie intake, improvement in quality of life, improved whole-body insulin sensitivity, decrease in glucose levels, shorter hospital stay
Nutritional interventions	
Low and very low calorie and ketogenic diet	Better weight reduction, visceral fat reduction, improvement in glycemic and lipid profiles, mean 30% reduction in liver volume
Pharmacotherapy	
GLP 1 receptor agonists	Higher T2DM remission rates, better body weight reduction, improvement in glycemic and lipid profiles
Psychological support	
Preoperative counseling and education	Reduced anxiety, depression, and fear, positive influence on eating disorders

GLP 1: Glucagon-like peptide 1; T2DM: Type 2 diabetes mellitus.

anti-obesity pharmacotherapy such as GLP-1 RA and in the future twincretins offers a significant opportunity to improve the peri and post-operative care of bariatric patients, acting in synergy with exercise and calorie-restrictive diets. Moreover, the degree of obesity and age influence the choice of treatment strategy or protocol in perioperative care. However, there are significant shortcomings as most of the research to date has been focused on the postoperative care of bariatric surgery patients, while research on perioperative care has been somewhat neglected.

CONCLUSION

We have attempted to summarize current knowledge and propose recommendations for perioperative care of all bariatric surgery patients, but with special emphasis on those with disturbances of glucose metabolism (Table 1). Future studies should focus on the development of perioperative treatment protocols consisting of the most optimal combination of lifestyle changes and pharmacotherapy thus optimizing response to bariatric surgery, ultimately improving both short -and long-term outcomes by reducing the incidence of T2DM and cardiovascular disease.

FOOTNOTES

Author contributions: Cigrovski Berkovic M conceived and wrote the original draft; Bilic-Curic I, Canecki Vrazic S, Mrzljak A and Cigrovski V were involved in data collection and analysis and writing the manuscript; all authors approved the final version of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Gong ZM
L-Editor: Webster JR

P-Editor: Gong ZM

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1106-1121

DOI: 10.4239/wjd.v13.i12.1106

ISSN 1948-9358 (online)

MINIREVIEWS

Diabetic foot ulcers: A devastating complication of diabetes mellitus continues non-stop in spite of new medical treatment modalities

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Mostafavinia A, Iran; Sivashanmugam K, India; Wu QN, China; Zhang LL, China

Received: July 21, 2022
Peer-review started: July 21, 2022
First decision: August 20, 2022
Revised: August 21, 2022
Accepted: November 18, 2022
Article in press: November 18, 2022
Published online: December 15, 2022

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Abstract

Diabetic foot ulcer is a devastating complication of diabetes mellitus and significant cause of mortality and morbidity all over the world and can be complex and costly. The development of foot ulcer in a diabetic patient has been estimated to be 19%-34% through their lifetime. The pathophysiology of diabetic foot ulcer consist of neuropathy, trauma and, in many patients, additional peripheral arterial disease. In particular, diabetic neuropathy leads to foot deformity, callus formation, and insensitivity to trauma or pressure. The standard algorithms in diabetic foot ulcer management include assessing the ulcer grade classification, surgical debridement, dressing to facilitate wound healing, off-loading, vascular assessment (status and presence of a chance for interventional vascular correction), and infection and glycemic control. Although especially surgical procedures are sometimes inevitable, they are poor predictive factors for the prognosis of diabetic foot ulcer. Different novel treatment modalities such as nonsurgical debridement agents, oxygen therapies, and negative pressure wound therapy, topical drugs, cellular bioproducts, human growth factors, energy-based therapies, and systematic therapies have been available for patients with diabetic foot ulcer. However, it is uncertain whether they are effective in terms of promoting wound healing related with a limited number of randomized controlled trials. This review aims at evaluating diabetic foot ulcer with regard to all aspects. We will also focus on conventional and novel adjunctive therapy in diabetic foot management.

Key Words: Diabetic foot ulcer; Peripheric artery disease; Macrovascular complications; Neuropathy; Wagner classification; Intralesionar growth factor treatment

Core Tip: Diabetic foot ulcers (DFU) are one of the most common problems and devastating complications of diabetes, and affect 15% of all diabetic patients and result in significant morbidity, mortality, and financial burdens. The management of DFU is usually complex and challenging to clinicians in clinical practice. This review article aims at summarizing the etiopathogenesis and classification of DFU. It also highlights novel adjunctive treatment modalities as well as conventional management for DFU.

Citation: Akkus G, Sert M. Diabetic foot ulcers: A devastating complication of diabetes mellitus continues non-stop in spite of new medical treatment modalities. *World J Diabetes* 2022; 13(12): 1106-1121
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1106.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1106

INTRODUCTION

Diabetic foot ulcers (DFU) are common clinical problems and devastating complications of diabetes, and affect 15% of all diabetic patients and results in significant morbidity, mortality, and financial burdens [1]. Five-year risk of mortality for a patient with diabetic foot ulcer is 2.5 times higher than the risk for a patient without[2]. Approximately 20% of moderate or severe DFU could cause some level of amputation. Moreover, 74% of them also have a risk of renal replacement therapy at 2 years[3]. This high mortality rate is also related with coexisting comorbidities such as cardiovascular or cerebrovascular diseases.

The pathophysiology of DFU is based on a triad of neuropathy, peripheral arterial disease, and concomitant secondary bacterial infection. Peripheral neuropathy could lead to intrinsic muscle atrophy and functional anatomical changes in the foot[4]. Eventually, progressive secondary foot infection penetrating deep facia, tendons, and joints could develop with repetitive inattention trauma. Infection could play a significant role for half of major lower limb extremity amputations. Recent studies indicate some risk factors for the development of DFU. These are as follows: Longer than 10 years of duration of diabetes, male gender, older patients, presence of comorbidities including nephropathy, neuropathy, and peripheral vascular disease, and history of foot ulceration[4-6].

The management of DFU is usually complex and challenging to clinicians in clinical practice. Costs of diabetic foot ulcerations have been increased to the treatment cost of many common cancers. Estimated costs of DFU management are greater than 1 billion in both developed and developing countries. Therefore, foot ulcers should be treated immediately by a multidisciplinary expert team for optimal outcomes. The treatment of DFU requires an immediate decision and systematic approach that comprises of maintaining arterial blood flow, treating the infection appropriately, and removing the pressure from the wound[7]. In addition, several adjuvant therapies are becoming a popular form of diabetic foot treatment. During the past 10 years, there has been an increasing amount of novel, basic science-based approaches and developments for adjuvant therapies including wound dressing, hyperbaric oxygen therapy, or growth factor formulations for efficient local delivery[8-10]. None of them had definitive results that improved wound healing and these approaches still need clinical validation.

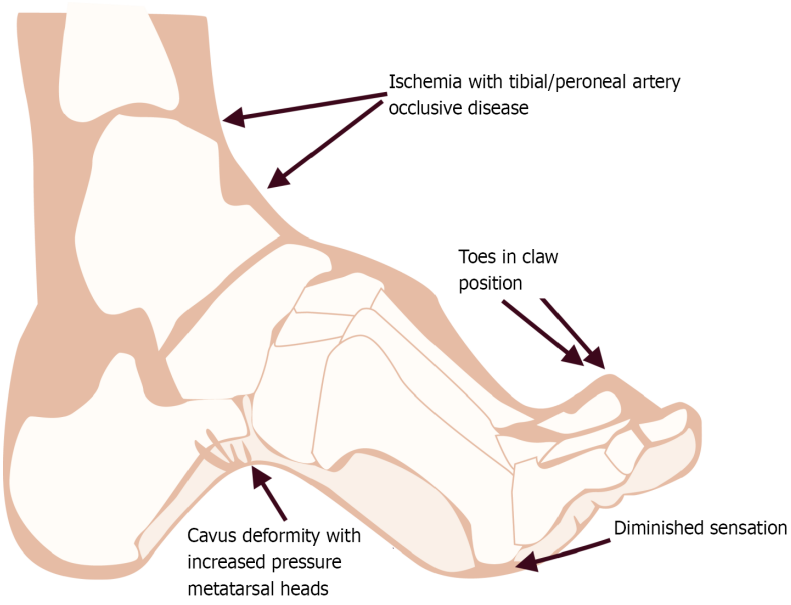
The review highlights novel adjunctive treatment modalities as well as conventional management for DFU.

PATHOPHYSIOLOGY

DFU is a serious result of risk factors including neuropathy, peripheral arterial disease, and secondary infection. Peripheral neuropathy could play a major role by producing intrinsic muscle atrophy and consequently leading to biomechanical anatomical changes on the feet such as hammer-toe formation, pes-planus, and pes-cavus, which lead to high-pressure zones of the foot[11,12] (Figure 1).

Diabetic neuropathy and foot ulcer

Diabetic neuropathy has multiple manifestations within the diabetic foot because it comprises sensory, motor, and autonomic fibers. The majority of patients with diabetes (66%) face peripheral neuropathy in the lower extremities[12]. Especially distal sensory neuropathy, the most type seen of all diabetic neuropathies, is a major risk factor for DFU but it is extremely variable, as it ranges from severely painful symptoms to a completely painless variety that may present with an insensitive foot ulcer[13]. Diabetic peripheral neuropathy mainly affects lower legs and the feet as a stocking-glowing distribution and it could cause the loss of the Achilles reflex which can be the first sign of these changes. The anatomy of the foot arch could change with the atrophy of the lumbricals and interosseus muscles and a



DOI: 10.4239/wjd.v13.i12.1106

Figure 1 Ethiopathogenesis of diabetic foot leading to vascular complications.

relative increase in the extensor tendon forces called "claw" deformity in the toes[14]. On the other hand, the onset of sensory neuropathy can manifest as loss of proprioception, pressure sensation, vibratory perception, and impaired gait[15]. Sensory neuropathy usually progresses gradually with the insidious appearance of symptoms that may be intermittent in the early stage. C-type fiber, which is a responsible for the sensorial transmission, results in inappropriate reaction to a painful stimulus[16]. Ulceration and infection could occur with repetitive trauma, and decreased sensation and proprioception could also predispose skin injury by producing atrophy and dislocation of protective plantar fat pads. Moreover sudomotor dysfunction could develop with diabetic autonomous neuropathy and it is also associated with foot ulceration due to dry skin and itching[17]. Several methods are developed to evaluate the sudomotor function. They are thermoregulatory sweat testing, quantitative sudomotor axon reflex, quantitative direct and indirect reflex test, and indicator plaster[18-20]. Among of them, indicator plaster represents a rapid, simple method based on color change from blue to pink at the plantar foot region.

Since defining neuropathic symptoms could be difficult due to fluctuation of the symptoms, a diagnosis of neuropathy may be difficult. Small fiber function can be determined by pinprick and a cold or warm thermal stimulus to the distal sensation. The proximal sensory abnormality could be determined by moving the individual test paradigms proximally. Positive stimulus (allodynia and hypersensitivity) for diabetic neuropathy, can be showed by measuring the intensity or area of these phenomena. Negative symptoms in diabetic neuropathy are also numbness, no sensation, poor balance, or muscle weakness. Clinical examination in these patients can include quantitative sensory testing (QST) to asses this sensory stimulus and also provide the advantage of directly assessing the degree of sensory loss in the foot. Some of the more commonly used techniques are "Semmes-Weinstein monofilaments", ''thermal and cooling threshold'', "perception of vibration", and "computer-assisted sensory examination"[21-23].

Semmes-Weinstein monofilament includes nylon filaments which have variable diameters and is one of the clinical tests that measure the response to a touching sensation of the monofilaments using a numerical quality. Inability to perceive pressure of 10 g (5.07) by the monofilament has been shown in subjects who are at risk for neuropathic foot ulceration (Figure 2). This is a very easy and applicable examination in busy outpatient clinics and reveals diabetic foot ulcer risk. It is recommended to all practitioners[24].

Perception of vibration, which is called deep sensation impairment, is usually one of the earliest signs of peripheral diabetic neuropathy. Tuning forks (128 Hz) are generally used in general practice; these determine the perception of vibration through the application on distal bony prominence of the great toe bilaterally and other bony prominences such as the medial malleolus. Sensitivity of tuning fork is approximately 53% and it is less predictive for the development of foot ulceration compared to using the monofilament test[25].

Although electrophysiology is not required for clinical diabetic neuropathy evaluation, it quantitatively assesses large fiber involvement in diabetic neuropathy. While these QSTs are convenient techniques in daily clinical practice, simple clinical instruments including Michigan neuropathy screening instrument and simplified neuropathy disability score can be used to assess neurologic

DOI: 10.4239/wjd.v13.i12.1106

Figure 2 Monofilament test is a diagnostic tool to detect diabetic peripheral neuropathy. When the nylon line bends, the force is 10 grams. It is used for diabetic foot contact and stress testing.

deficits. Now these scoring systems become using in a number of ongoing trials with new therapies for diabetic neuropathy[26,27].

Although it is not the main topic of this review, briefly neuropathy management is done symptomatically and current therapeutic agents which are used, including tricyclic antidepressants, serotonin and noradrenalin reuptake inhibitors, and anticonvulsants, have efficacy in diabetic neuropathy[28]. Among these new anticonvulsants, pregabalin and gabapentin have been shown to be more convenient in the treatment of painful syndromes in recent articles[29].

Callus deformity and plantar shear stress

Calluses have been defined as hyperkeratosis caused by excessive mechanical loading. Calluses increase pressure of plantar area and the risk of DFU[30]. Significant risk factors for callus deformity in patients with diabetic neuropathy are foot deformity, limited joint mobility, repetitive stress of walking, and ill-fitting shoes[31]. Calluses are frequently developed under bony prominence including the metatarsal head. Proprioceptive loss due to sensory neuropathy and metatarsal heads leads to increased pressure and load under the diabetic foot. It has been reported that callus deformity may be related with a relative risk of 11 for ulcer development (Figure 3). As a result, removal of plantar callus is associated with reduced plantar pressure and thereby reduced foot ulcer risk.

Charcot neuroarthropathy

Charcot neuroarthropathy (CN) has been determined to be a chronic and destructive disease of the bone structure and joints in patients with neuropathy. The precise incidence of CN in persons has previously been estimated to be between 0.1%-0.4%[31]. Etiopathogenesis of CN is complex and based on varied degrees of neuropathy. Typical clinical symptom is characterized by painful or painless bone and join destruction in limbs that have lost sensory innervation. Although the clinical management of CN has many clinical challenges, it is generally characterized with asymptomatic nature such as ankle sprain, cellulitis, and thrombosis[32]. A diagnosis of CN is based on primarily on thorough history and physical examination, with corroborating laboratory investigations and diagnostic imaging. Modified eichenholtz classification is commonly used for description in the clinical stage[33] (Figure 4). According to this classification, stage 0 is mild inflammation, soft tissue edema, and normal X-ray, stage 1 is severe inflammation and abnormal X rays with microfractures, stage 2 is coalescence and end of bone resorption, and stage 3 is definitive bone remodeling with chronic CN.

Initial weight-bearing radiography can show demineralization, fragmentation, joint dislocation, osseous debris, and joint space obliteration[34]. But routine radiography gives limited information about the differentiation of CN from osteomyelitis. Imaging techniques including magnetic resonance imaging (MRI) or PET/CT scans highlight the inadequacies of clinical examination and radiographs in assessing the CN stage[35]. Orthopedic surgery is often required to correct severe foot deformities when conservative measures including physiotherapy are not effective (Figure 5).

Peripheral vascular disease

Peripheral vascular disease is characterized as a chronic arterial occlusive disease of the lower extremities and varies in severity. Patients with peripheral arterial disease have usually intermittent claudication, rest pain, and tissue loss with or without gangrene[36]. Rest pain is shown in these patients and related with chronic sensory nerve ischemia. Resting pain emerges as a diffuse burning or

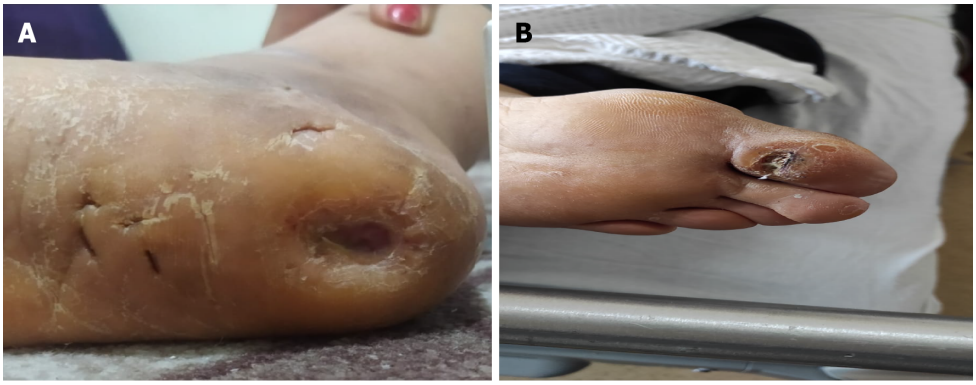


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Figure 3 Callus formation as a presentation of diabetic neuropathy.

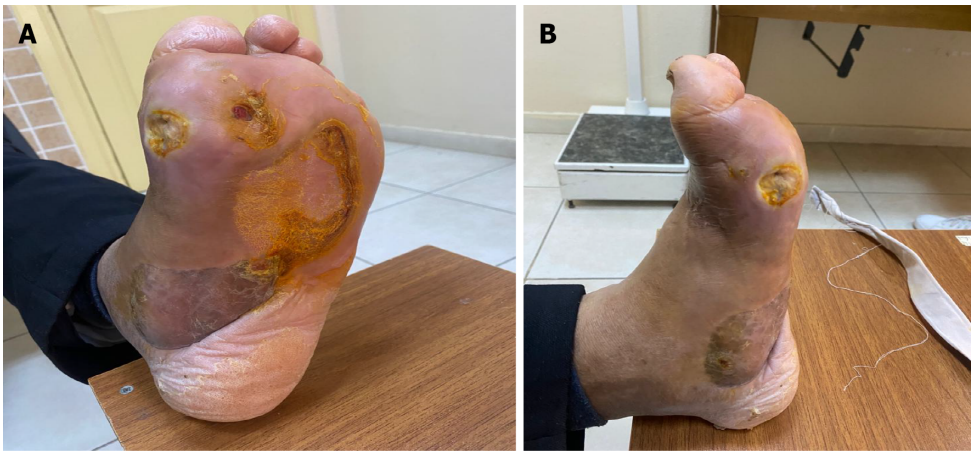

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Figure 4 Charcot neuroarthropathy is a chronic devastating and destructive disease of bone structure and joint in patients with neuropathy. A and B: Rocker-bottom foot deformity to charcot process.

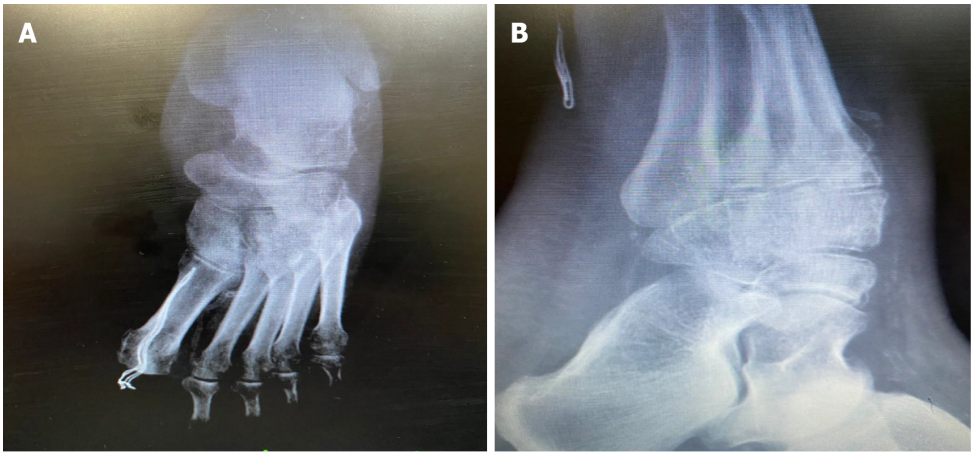

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Figure 5 Radiographic findings. A and B: Radiographic findings of charcot neuroarthropathy.

aching pain in the fore foot[37]. Intermittent claudication and rest pain are the clues to diagnosis, though it can lack symptoms or can be difficult to be attributed exclusively to peripheral vascular disease. Any worsening of walking quality or speed should be taken into account, as well as fatigue, pain, cramps, discomfort, or burns in the buttocks, thighs, or feet. The extent of ischemia and symptoms are related with location vascular lesion as well as the development of collateral circulation[38].

Generally, patients with aorto-iliac disease have buttock and thigh pain, and femoral disease causes calf discomfort. Tibio-peroneal disease generally does not have claudication, although some patients will complain of foot pain or numbness while walking.

Pulse palpation (distal pedis, posterior tibial, posterior tibial, popliteal, and femoral arteries), which can be a simple, cheap, and comfortable clinical examination, should be performed on all diabetic patients during the follow-up examination. Ankle-brachial index (ABI) has emerged as a relatively non-invasive tool for the diagnosis of peripheral arterial disease[39]. The measurement of ABI (ratio of systolic blood pressure on the ankle to the systolic blood pressure in the upper arm) is normal in the 1.0-1.4 range, obviously pathologic under 0.9. An ABI over than 1.4 is also considered as abnormal, reflecting calcified and stiffed arteries. Doppler ultrasound examination and computed tomography angiography are often used as non-invasive tests[40]. Intra-arterial digital subtraction angiography is defined as the gold standard for arterial imaging because of its high spatial resolution. It has the advantage of allowing endovascular therapy during the same procedure and it is also extremely accurate for the smaller vessels of the ankle and foot. But it has a disadvantage for patients with renal insufficiency [glomerular filtration rate (GFR) < 35 mL/min/1.73 m^2][37].

Infection and osteomyelitis

Infection in ulcerated diabetic foot is a primary cause of morbidity and mortality. It is well known that diabetic patients are prone to infectious diseases because of the diminished host defenses, including inadequacy in leukocyte capacity and morphologic alterations to macrophages, elevation of proinflammatory cytokines, and impairment of diabetic polymorph-nuclear cell functions (chemotaxis, phagocytosis, and killing)[41,42]. Many organisms can cause diabetic foot infection, but Gram positive cocci, especially *staphylococci* (*S. aureus*), are the most common cause pathogens[43]. Peripheral neuropathy, angiopathy, and a lack of attention to foot hygiene such as using poorly fitting footwear are the major factors in the development of infection. Abrasions, rashes, and loss of skin integrity due to fungal infection can be facilitating factors for diabetic foot infection. Approximately 60% of foot infections starts in webbed spaces and 30% in nails, while 10% are secondary punctures[44]. Ulcers > 60 mm^2 in size, purulent discharge from sinus tract, presence of sausage toe, and erythrocyte sedimentation rate > 70 mm/h suggest the presence of underlying osteomyelitis[45]. Osteomyelitis could be able to occur after the spread of superficial infection of the soft tissue on the adjacent bone or marrow. Although numerous expensive radiology techniques are available to diagnose osteomyelitis, specific clinical signs of inflammation (swelling, erythema, warmth, tenderness, pain, or induration) and the use of simple metal probes can often be used to make clinical diagnoses. In initial clinical visit, plain radiographs should be obtained to determine the extent of osseous erosion, as well as to assess anatomy for surgical planning. Further scanning tests including MRI and bone scans could be performed for patients with neuropathic osteoarthropathy or multifocal disease[46-48].

CLASSIFICATIONS OF FOOT ULCERS

Identification and classification of patients with DFU should be performed to see whether hospitalization, intravenous (IV) broad spectrum antibiotics, or surgical consultations will be required or not. An accurate defining of ulcer characteristics such as size, depth, appearance, and location allows for the mapping of progress during management of DFU. There have been several classification systems that have been broadly externally validated for ulcer healing and lower extremity amputation, and they are Meggitt-Wagner, University of Texas, Infectious Disease Society of America (IDSA), perfusion, extent, depth, ischemia, sensation (PEDIS), SINBAD, and Wound, Ischemia and foot Infection (WIfI) classification[49-51].

The Meggitt-Wagner classification was the first announced classification system; however, it is not well validated and does not distinguish well between ulcer types for the main purpose of classification. This system consists of six different groups: 0, intact skin; 1, superficial ulcer; 2, ulcer reaching to tendons, joints, bones; 3, deep ulcer with abscess and osteomyelitis; 4, local gangrene; and 5, gangrene of the entire foot. It presents vascular perfusion only when gangrenous changes appear and infection when osteomyelitis is present (Table 1).

The University of Texas classification is well validated but it does not indicate neuropathy or depth of the ulcer area, which is considered to be one of the main determinants of the ulcer healing (Table 2). IDSA was reported as a guideline and diabetic foot is subclassified into the categories of uninfected, mild (restrictive involvement of only skin and subcutaneous tissue), moderate (more extensive), and severe (systemic signs of infection).

The PEDIS classification system is based on five features of the wound and it helps clinicians assess risk or prognosis for a person with diabetes and active foot ulcer. In addition, it was used for a clinical audit study in 14 European countries[52].

The SINBAD classification system matches each composing variable such as area, depth, infection, and neuropathy to a score (ranging from 0-6). This classification system has some benefits such as simple, quick to use and not requiring specialist equipment[53].

Table 1 Wagner-Meggit classification

Grade	Lesion
0	No open lesion
1	Superficial ulcer
2	Deep ulcer to tendon or joint capsule
3	Deep ulcer with abscess, osteomyelitis or joint sepsis
4	Local gangrene - fore foot or heel
5	Gangrene of entire foot

Table 2 University of Texas Classification system

	0	1	2	3
A	No open lesion	Superficial wound	Affected tendon/capsules	Affected bone/joint
B	With infection	With infection	With infection	With infection
C	Ischemic	Ischemic	Ischemic	Ischemic
D	Infection/ischemia	Infection/ischemia	Infection/ischemia	Infection/ischemia

The WIfI classification includes three prognostic factors that affect clinical management. These factors indicate wounds that are graded from 0 to 3, ischemia graded based on toe pressure index, and infection which is based on IDSA classification. The WIfI threatened limb classification has been shown to correlate well with a risk of major amputation but it is not enough make acute decisions about the treatment only by itself due to more confounding factors (Table 3).

Up to date there has been several classification systems but which classification to use is still controversial. Physicians who treat patients with DFU are concerned about which classification is recommended. They are still discussing the usefulness of these classifications, and the effects of such classifications on diagnosis or treatment remain unknown. Although there are some limitations in case of prognostic accuracy of the Meggitt-Wagner classification, this classification remains the most commonly utilized system in health care today.

MANAGEMENT OF DIABETIC FOOT ULCER

The main principle of management of DFU is to evaluate wound appearance (extent, size, depth, presence of infection, and wound duration) in detail. Clinicians should inspect the extent of tissue destruction and possible bone and joint involvement. After evaluating wound appearance, another major decision is whether the patient can be initially treated as an outpatient or needs hospitalization. Early superficial ulcer (< 2 cm) without systemic toxicity may be treated at home. If the patient has a deep gangrenous ulcer with infected or systemic symptoms or needs surgical treatment, hospitalization is advised.

Since diabetic ulcer healing depends on multiple factors, it should be evaluated by a multidisciplinary expert team. The treatment includes conservative and surgical interventions and there are some fundamental steps of diabetic foot management such as surgical debridement, dressing, wound offloading, vascular assessment, control of infection, glycemic control, and adjuvant therapies[54-57] (Table 4).

Surgical debridement

Debridement is a principal treatment of local wound healing and it involves removing hyperkeratotic epidermis (callus), necrotic dermal tissue, foreign debris, and bacterial elements from a wound bed. Debridement includes numerous forms such as mechanical, autolytic, enzymatic, and sharp[58,59]. Sharp debridement is more common to use, and it includes two forms, namely, clinic based debridement and surgery based excisional debridement. A combination of debridement methods could help to remove devitalized tissue that provides a nidus for bacterial proliferation and acts as a physical barrier for antibiotics. Debridement is the most important step of the wound healing. If necessary, it should be performed in every clinic visit by clinicians.

Table 3 Wound, Ischemia, and foot Infection classification

	Wound	Ischemia; toe pressure/*tcpo*$_2$	Infection
0	No ulcer and no gangrene	> 60 mm/Hg	Non-infected
1	Small ulcer and no gangrene	40-59 mm/Hg	Mild (< 2 cm sellulitis)
2	Deep ulcer and gangrene limited to toes	30-39 mm/Hg	Moderate (> 2 cm sellulitis)
3	Extensive ulcer or extensive gangrene	< 30 mm/Hg	Severe (systemic response/sepsis)

Table 4 Standard care of diabetic foot ulcer

Treatment	Description	
Debridement	Surgical debridement	Necrotic or non-viable tissue should be removed, regular (weekly) debridement is associated with rapid healing of ulcers
Dressing	Films, foams, hydrocolloids, hydrogel	Proper using of dressing materials could facilitate moist environment
Wound off-loading	Rock or bottom outsoles, custom-made insoles, some shoe inserts	Plantar shear stress should be removed
Vascular assessment	PTA or endovascular recanalization followed by PTA or by-pass grafting	Arterial insufficiency should be treated for improving wound healing
Control of infection	Appropriate antibiotic therapy according to pathogens	Deep tissue cultures should be obtained before antibiotic therapy, for mild infection treatment duration could be 1-2 wk but for moderate to severe infection, it should be 3-4 wk
Glycemic control		For better glycemic control, insulin treatment has been preferred in hospitalized patients with diabetic foot ulcers

PTA: Percutaneous transluminal angioplasty.

Dressings

After the adequate debridement period, soft tissue defect requires dressing materials for closure and/or coverage of wound area. Dressing with adequate biomaterials could provide wound healing processes and protect from contamination. Naturel skin is considered perfect wound dressing and therefore ideal wound dressing should try to mimic its properties. Since recent studies highlighted the role of wound environment, dressing also should be biocompatible and not provoke any allergic or immune response reaction and should be easily removed[60,61]. Alginate and collagen-alginate products, carboxymethyl-cellulose dressings, topical phenytoin, and hydrogels are types of dressings which are available[62,63]. But there have been still some questions to support the choice of any dressings or to promote healing of ulcer.

Wound off-loading

Plantar shear stress and vertical plantar pressure are major causative factor in the development and poor healing of DFU. Removal of pressure and/or redistribution of an increased weight bearing area of the foot can be achieved through off-loading strategies. Total contact casts and removable walkers are used for off-loading the diabetic foot[64]. Various therapeutic off-loading devices such as rock or bottom outsoles, custom-made insoles, and some shoe inserts (*e.g.*, metatarsal pads and medial arch supports) may reduce fore foot peak pressure[65,66]. Recently, The International Working Group[67] on the diabetic foot suggests the following recommendations: (1) Removal of pressure on ulcers is one of the main part of the treatment plan; (2) non-removable walkers are the preferred treatment; and (3) forefoot off-loading shoes or cast shoes may be used when above-the-ankle devices are contraindicated.

Vascular assessment

Revascularization of critically ischemic legs results in increased area perfusion after the procedure, which is in turn associated with a further reduced amputation rate. Arterial revascularization can be performed through open procedures such as a bypass or, in many cases, endovascular recanalization followed by PTA (percutaneous transluminal angioplasty) with or without adjunctive stenting[68]. Overall, the aim of vascular reconstruction is to restore direct pulsatile flow in at least one or more arterial structures, preferably feeding the wound.

Control of infection

The diagnosis of infection is based on parameters of inflammation and should always be classified

according to a preferred classification method. Antibiotic therapy is based on possible pathogens, presence of vascular disease, and the extent of foot infection. Hospitalization with parenteral antibiotic treatment is recommended when the infection penetrates to the deep fascia. Patients with chronic ulcer, prior antibiotic treatment, and recurring infection should be assumed to have methicillin-resistant *Staphylococcus Aureus* infection[69]. The spectrum can be broadened to cover Gram-negative aerobes in chronic infections. If a patient has a superficial ulcer without infection, empiric antibiotic treatment therapy is not recommended[70]. Oral therapy including trimethoprim/sulfamethoxazole or amoxicilline/clavulanic acid plus linezolid is recommended to patients with a superficial ulcer and presence of pedal pulses. Parenteral therapy such as vancomycin or daptomycin plus piperacillin/tazobactam or imipenem cilastatin or meropenem has been recommended to patients with systemic inflammation or ulcer/gangrene with penetration of deep fascia[71,72]. In addition, in order to avoid antibacterial resistance or other adverse outcome of therapy, it is the best approach to be followed.

Glycemic control

In hospitalized patients, intensive insulin treatment should be administered to patients with foot ulcer for better glycemic control. The evidence of glycemic control can accelerate the healing of foot ulcers and reduce the incidence of ulceration and amputation. Studies have shown that increased blood glucose level is correlated with decreased neutrophil function and suppression of inflammatory response[73-75]. Although there are limited randomized control trials (RCTs) which determine whether glycemic control improves wound healing better, in a meta-analysis which included 10897 patients without known history of peripheral arterial disease, it has been reported that intensive glycemic control was associated with a statistically significant decrease in risk of amputation of diabetic foot[76].

Adjuvant therapies

New treatment modalities have been developed since 2002. There are advanced wound therapy methods. In addition to conventional therapy, several types of treatment modalities are available, such as negative pressure wound therapy, which is also known as vacuum assisted closure (VAC), synthetic skin grafts, non-surgical debridement agents, topical growth factors, electrical stimulation, and hyperbaric oxygen chambers[77-83] (Table 5). Each has its own merits but economic constraints and patient compliance should be kept in mind.

Negative pressure wound therapy

Pressure wound therapy (VAC) was first declared and used in clinical practice by the German physician Fleischmann in 1993[77]. It has remarkable effect on wound drainage, also enhancing perfusion. VAC could be used for acute and chronic DFU and it promotes the growth of granulation tissue (Figure 6). It can be helpful in the postoperative management of DFU. According to the Wound Healing Society and European Wound Management Association[78]: (1) Wound infection should be well controlled after the debridement; (2) the risk of bleeding is well controlled; there is no active bleeding or exposed vascular damage on the wound or no risk of coagulation dysfunction; and (3) the risk of ischemia is treated and well controlled (ABI ranges from 0.9 to 1.3), and VAC therapy is recommended with class 1 evidence. After 1-2 rounds of VAC therapy, a comprehensive evaluation should be performed for an effective evaluation. It is recommended to continue VAC if there is growth of new granulation tissue on the wound surface with surrounding epithelization. If infection is aggravated, it should be stopped immediately. In recent years, VAC treatment has been used extensively for DFU management and studies have shown that it has certain advantages in preventing and controlling wound infections.

Synthetic skin grafts

Skin substitutes are classified into three groups based on the plasticity of preparation procedure and composition of the substitutes. Class 1 skin layer includes cultured epidermal equivalents which are formed of single-layered materials; Class 2 layer includes dermal components from processed skin or fabricated matrix protein; Class 3 layer also consists of dermal and epidermal components and skin grafts (allograft and xenograft)[80]. Class 3 layer is more popular and common to use. Although there are limited studies, wound healing can be promoted by using these agents.

Non-surgical debridement agents

Although sharp debridement can play a major role in wound healing, various techniques such as autolytic debridement with hydrogels, enzymatic debridement, maggot and larval debridement, and hydrotherapy are available[81,82]. But recent studies did not provide sufficient evidence to use one approach over other methods.

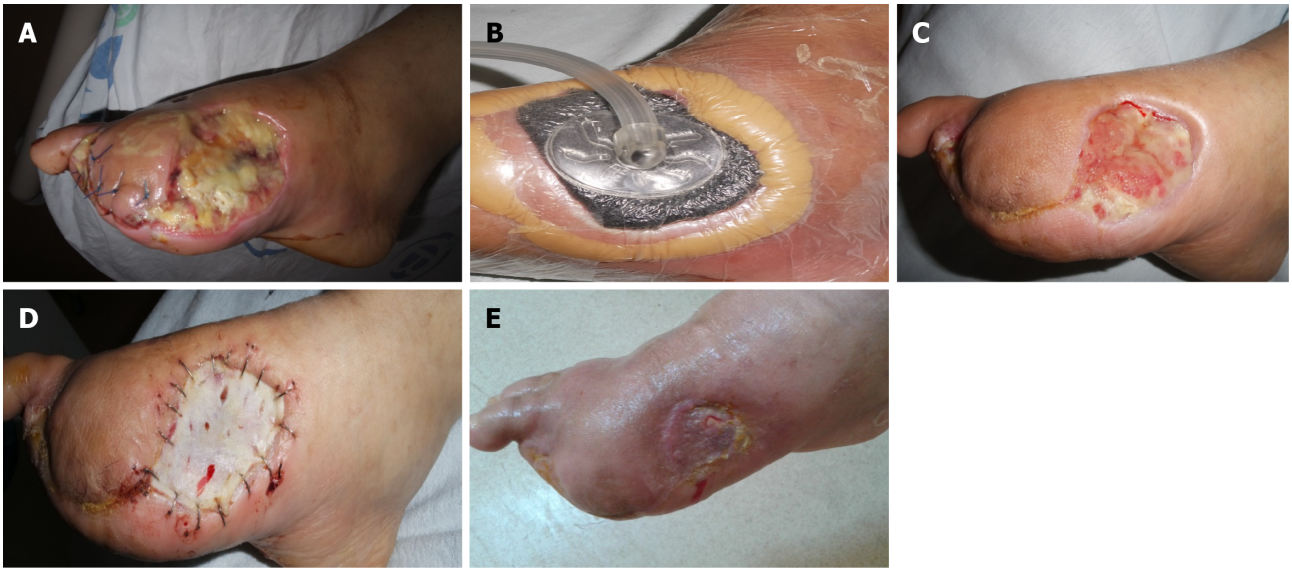
Hyperbaric oxygen chamber

Administration of 100% O_2 has some beneficial effects on wound healing. It not only causes increased blood and oxygen content in hypoxic tissue, but it also has antimicrobial activity due to enhanced mobility and bacteriophagic activity of leukocytes[83]. Studies show that hyperbaric oxygen stimulates

Table 5 Additional adjuvant care of diabetic foot ulcer

Item	Description
Negative pressure wound therapy (VAC)	Widely used, removal of the excess third space fluid from the area, reduction of bacterial load, increased granulation tissue, but RCTs have high risk of bias
Synthetic skin grafts (Bio-engineered skin substitutes)	Contribute to the new dermal tissue but limited data to prove benefit of these products
Non-surgical debridement agents (enzymatic debridement, autolytic debridement, hydroterapy, Maggot therapy)	Promoting fibroblast migration and improving skin perfusion but due to small RCTs, it has clinical bias for beneficial effect
Topical growth factors (EGF, VEGF, PDGF, FGF)	Promote healing non-infected foot ulcer and stimulating angiogenesis but limited trials confirming positive outcomes
Electrical stimulation	Bacteriostatic and bactericidal effect on foot ulcer but lack of evidence due to limited clinical trials
HBOC	HBOC therapy increases blood and oxygen content in hypoxic tissues and has antimicrobial activity, but it is unclear whether it has benefit in long term wound healing

RCT: Randomised controlled studies; HBOC: Hyperbaric oxygen chambers; VAC: Vacuum assisted closure; EGF: Epidermal growth factor; VEGF: Vascular endotelial growth factor; PDGF: Platelet derived growth factor; FGF: Fibroblast growth factor.



DOI: 10.4239/wjd.v13.i12.1106

Figure 6 Negative pressure wound therapy is considered as a better alternative therapy for the management of diabetic foot ulcer. A-E: This patient was treated with negative pressure wound therapy therapy after surgical therapy.

angiogenesis and increases fibroblast proliferation and collagen production. Some authors suggested that there are no definite results which display an improvement in DFU. There is large uncertainty associated with the evaluation of the cost-effectiveness of hyperbaric oxygen therapies. Up to date, there have been seven RCTs showing that hyperbaric oxygen chambers are beneficial for preventing amputation and promoting complete healing in patients with Wagner grade 3 or greater DFU[84,85]. In patients with Wagner grade 2 or lower DFU, there is inadequate evidence to justify the use of hyperbaric oxygen therapy as an adjuvant treatment. The most common adverse events associated with hyperbaric oxygen therapy are barotraumatic otitis, the inability to equalize middle ear pressure, and worsening of cataracts.

Topical growth factors

Epidermal growth factor (EGF), ascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) are polypeptide growth factors that have significant effects on tissue repair processes. These growth factors are released by platelets and activated macrophages that are required for normal wound repair[10,86]. Growth factors which are used topically were reported to reduce the incidence of lower limb amputation. Using of growth factors is essential to promote angiogenesis, enzyme production, and cell migration and proliferation.

PDGF is a crucial factor in wound healing and serves as a chemo-attractant for the migration of fibroblasts and neutrophils to the site of injury. It is the first recombinant growth factor approved for topical application of wound healing. Fewer RCTs evaluated the effectiveness of PDGF and all studies applied the topical gel with different concentrations[87,88]. The majority of them did not find significant healing improvements compared to the standard wound care.

FGF acts as a balance factor in the body and is important for tissue maintenance, repair, regeneration, and metabolism. FGF is a stronger angiogenesis factor than PDGF and VEGF. And FGF stimulates angiogenesis and proliferation of fibroblasts, forming granulation tissue. FGF has some limitations for the treatment of DFU wound healing, since it generally has a short half-life and require repeated administrations[89].

EGF is a wound modulator that is involved in cell migration and proliferation. Injecting EGF deep into the wound bottom and contours encourages a more effective pharmacodynamic response in terms of granulation tissue growth and wound closure[90,91]. EGF is perhaps the most widely used method in diabetic foot wound therapy but the results of studies are controversial or neutral. But our clinical experiences have shown that EGF is promising for healing foot ulcer (Figure 7).

Low level laser therapy

Low level laser therapy (LLLT) is a novel adjunctive therapy and is known to supply direct biostimulative light energy to body cells. This energy could stimulate molecules and atom of cells but does not cause a significant increase in tissue heat. Although different laser wavelengths have different depth of penetration of tissue, red laser has a deeper penetration than the others such as violet, blue, green, and yellow[92]. LLLT, which can be considered as a possible new treatment option for the diabetic foot, has a various effect on wound healing by cellular migration or penetration[93]. Otherwise clinical trials using human models do not provide sufficient evidence to establish the uselfulness and practical method in wound care regimes.

Although these newly adjunctive treatments have some benefits, they are costly and should be reserved for ulcers that fail to respond to standard treatments. Adjunctive treatment modalities should be considered as an addition to good wound care which must always include adequate off-loading and debridement therapy. Current evidence points towards VAC therapy and local stem cell application as an effective treatment than the other adjunctive modalities for diabetic foot healing. There is a need for well-designed blinded RCTs to determine the true efficacy of these interventions and to develop evidence-based practice guidelines.

PREVENTION OF FOOT ULCERATION

DFU are a devastating complication of diabetes mellitus. The mainstay of diabetic foot intervention is prevention. Preventative strategies in the form of education and regular foot assessments for peripheral vascular diseases and neuropathy along with risk stratification form the basis of the management of diabetic foot disease. Recently published guidelines highlight risk stratification for the assessment of risk for diabetic foot ulcer or risk of future amputation[55,94]: Very low risk: No loss of protective sensation and no peripheral arterial disease; Low risk: Loss of protective sensation or peripheral arterial disease or presence of callus formation alone; Moderate risk: Loss of protective sensation and peripheral arterial disease or presence of foot deformity; High risk: History of previous ulceration or previous amputation or renal replacement therapy or neuropathy and non-critical ischemic neuropathy with callus.

According to IWGDF prevention guidelines, it is recommended to examine a person who has a low risk of foot ulceration annually for signs or symptoms of loss of protective sensation and peripheral arterial disease. Patients with moderate or high risk should be screened every 3-6 mo. A person's risk status may change over time, thus requiring continual monitoring. Patients who have a risk of foot ulceration should be informed about controlling the whole surface of boot feet and the inside of shoes daily. Patients with moderate or high risk should be warned about wearing proper footwear to reduce plantar pressure. If there is a pre-ulcerative sign such as callus, appropriate treatment should be performed. Achilles tendon lengthening, joint arthroplasty, and single or pan metatarsal head resection may be considered for patients who cannot heal with non-surgical therapy.

If the preventative treatment modalities are carried out for patients with diabetes, the global patient and economic burden of diabetic foot disease can be considerably reduced. Decreasing the risk of ulceration also reduces the risk of infection, hospitalization, and lower extremity amputation in these patients.

CONCLUSION

DFU constitute a substantial burden for all over the world. Optimized therapy requires a collective

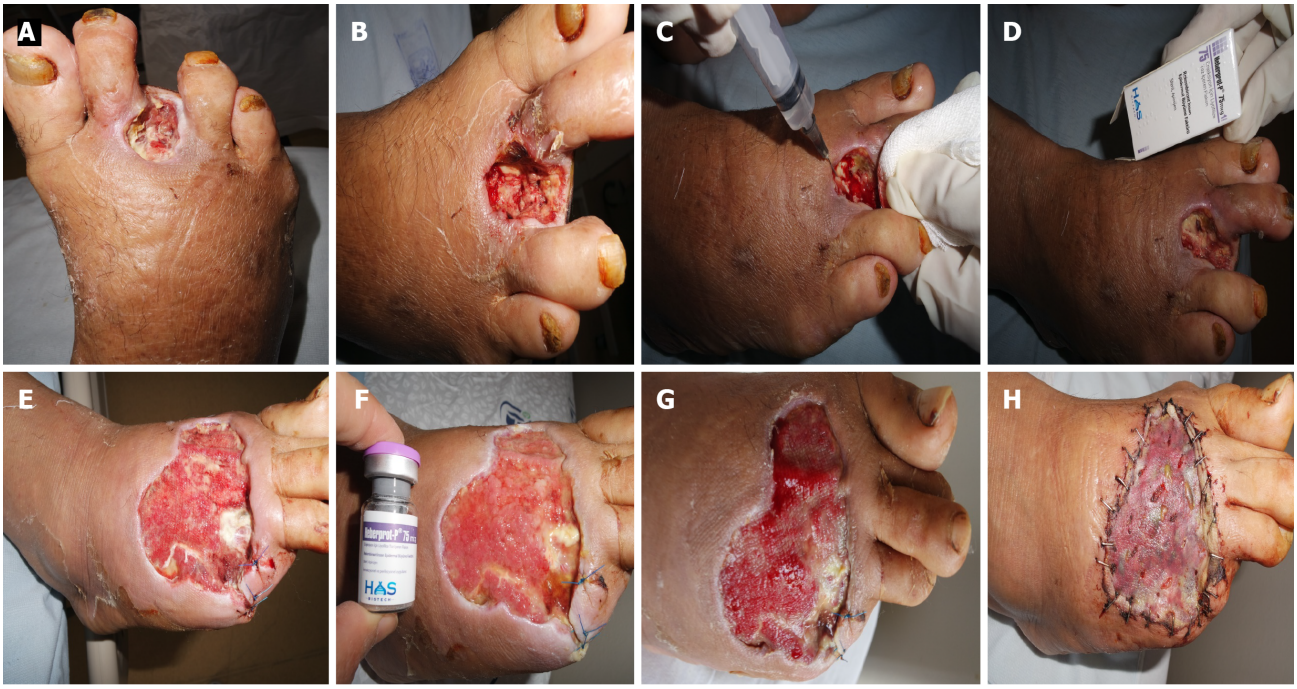


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Figure 7 Epidermal growth factor is perhaps the most widely used method in diabetic foot. A-D: Intralesional epidermal growth factor therapy into the wound bottom and contours encourages granulation tissue growth and wound closure; E-H: Before and after intralesional epidermal growth factor therapy.

refocusing on prevention and reallocation of resources from simply healing active ulcers. Multidisciplinary expert team is necessary for management of complex DFU and therefore multidisciplinary approach to patient care reduces the risk of amputation in patient with DFU. A combination of care from vascular, cardiovascular, infectious disease, and endocrinology disciplines as well as podiatrists and wound care specialists provides a full range of care for patients with DFU. Conventional therapies including debridement, off-loading, vascular assessment, and control of infection are principal treatment modalities. Otherwise, better outcomes could be obtained when the conventional treatment is combined with additional treatment in suitable patients.

FOOTNOTES

Author contributions: Akkus G contributed to data collection and writing; Sert M contributed to review.

Conflict-of-interest statement: There are no conflicts of interest to report.

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S-Editor: Chen YL
L-Editor: Wang TQ
P-Editor: Chen YL

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World Journal of Diabetes

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World J Diabetes 2022 December 15; 13(12): 1122-1130

DOI: 10.4239/wjd.v13.i12.1122

ISSN 1948-9358 (online)

MINIREVIEWS

Hyperbaric oxygen therapy and chemokine administration - a combination with potential therapeutic value for treating diabetic wounds

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Oley MH, Indonesia; Samadi N, Iran

Received: August 22, 2022
Peer-review started: August 22, 2022
First decision: September 12, 2022
Revised: September 30, 2022
Accepted: November 4, 2022
Article in press: November 4, 2022
Published online: December 15, 2022

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Abstract

Non-healing wounds impart serious medical problems to people with diabetes. Amongst 15% of diabetic patients, the incidence of foot ulcer is the most prevailing, which confers a significant risk of limb amputation, mainly due to hypoxia and impairment in cell signaling. Alteration in the expression of chemokines and the related factors in diabetic conditions delays the recruitment of different cell types, including fibroblasts, keratinocytes, and immune cells such as macrophages to the site of injury, further impairing neovasculogenesis, re-epithelialization, and extracellular matrix formation. Thus, proper activation of effector cells through an accurate signal pathway is necessary for better therapeutic application. Hyperbaric oxygen therapy (HBOT) is the current treatment prescribed by medical practitioners, shown to have increased the wound healing rate by reducing the need for significant amputation among the diabetic population. However, the risk of morbidity associated with HBOT needs complete attention through rigorous research to avoid adverse outcomes. Altering the level of pro-angiogenic chemokines may regulate the inflammatory response, further promote vascularization, and enhance the complete healing of wounds in diabetic patients. Thus, a combination of better therapeutic approaches could pave the way for developing a successful treatment for diabetic foot and wound healing.

Key Words: Diabetic foot; Wound healing; Hyperbaric oxygen therapy; Chemokines; Combinatorial therapy

Core Tip: Diabetes induces slow or non-healing of wounds, increasing the risk of developing infection and other complications. Proper management of blood sugar levels is essential to improve the overall health. Hyperbaric oxygen therapy (HBOT) enhances the efficacy of wound healing rate in chronic diabetic foot ulcer patients. However, the systemic and meta-analysis data contradicts in cases associated with ischemic wounds. Also, the uncordial functioning of effector cells due to the interrupted signaling pathway involving chemokines and related growth factors worsens the condition of wound healing to a greater extent. Thus, a combinatorial approach of HBOT and chemokine administration could have potential therapeutic value for treating diabetic wounds with the existing clinical protocol.

Citation: Venkataseshan J, Viswanathan P. Hyperbaric oxygen therapy and chemokine administration - a combination with potential therapeutic value for treating diabetic wounds. *World J Diabetes* 2022; 13(12): 1122-1130
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1122.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1122

INTRODUCTION

Diabetic patients often encounter non-healing or improper healing of wounds, which is a serious medical problem. Amongst many complications imparted by diabetes, the incidence of diabetic foot ulcer (DFU) is the most prevailing, significantly increasing the risk of limb amputation in 25%-90% of diabetic patients if the proper medication is either not provided or followed[1]. It has been estimated that the current incidence of DFU will affect 15% of all patients related to diabetes[2]. DFU is an open sore or wound that generally begins from minor trauma, pressure, or irritation at the bottom of the foot. The morbidity of DFU leads to chronic pain, suffering, and poor quality of life for diabetic patients. The changes in the biomechanics of bones and architecture of soft tissues increase the risk of atherosclerotic arterial diseases and peripheral neuropathy, which could lead to nonenzymatic glycation predisposes causing ligament stiffness and decreased nerve sensation. Due to this, the patient would be unaware of pain on the foot or lower limb[3]. Also, prolonged hyperglycaemia impairs the function of immune cells, making the wound prone to infections. Thus, the overall physiological impairment associated with DFU complicates wound healing and detains precise treatment due to the lack of evidence-based protocol[4]. Although trauma and neuropathy are the critical triads for developing DFU, studies have shown that an intricate signaling mechanism is involved at the molecular level[5].

Wound healing is a cellular response which involves numerous processes such as hemostasis, inflammation, keratinocyte proliferation, angiogenesis, vascular epithelialization, fibroblast differentiation, collagen production, and tissue remodeling. However, oxygen perfusion to the site of injury is crucial for an effective outcome. Hypoxia, a state of low oxygen tension, induces cellular stress through a complex cascade by delaying the recruitment of pro-inflammatory cells, impairing growth factor expression, and resulting in defects in angiogenesis and extracellular matrix (ECM) formation[6]. Evidence suggests that diabetes induces hypoxia in the tissues of the kidneys, retina, adipose, and skin-related wounds[7]. Hypoxia-inducible factor (HIF) is the key transcriptional regulator that plays a prime role in the adaptive response to oxygen homeostasis. In the presence of oxygen (optimal concentration), HIF undergoes hydroxylation and subsequent ubiquitination to degrade in a shorter time. However, under hypoxic conditions, HIF undergoes stabilization and translocates to the nucleus to regulate the activation of genes associated with glucose metabolism and angiogenesis[8]. Studies showed that hyperglycemia destabilizes HIF and dysregulates downstream transcriptional activation, resulting in disease progression. However, the exact mechanism is still unknown[7].

In hyperbaric oxygen therapy (HBOT), a patient is treated by delivering 100% oxygen under a supra-atmospheric pressure. Evidence suggests that providing HBOT to patients suffering from Wagner grade 3 wound or higher DFUs during the postoperative period has greatly reduced the risk of limb amputation and incomplete re-epithelialization[9]. However, the clinical practice guidelines recommend against using HBOT for patients with Wagner grade 2 or lower DFU as the chance of oxygen toxicity is higher[10]. Thus, more research-based evidence is needed for effective treatment to prevent morbidity and mortality. Although HBOT is currently approved and recommended by the Centre for Medicare Studies for treating DFUs, the management remains complex[9].

Chemokines are signal molecules that play a crucial role in coordinating the activation and migration of immune cells to the site of injury[6]. The cytokines and growth factors produced by the immune cells promote wound healing during the inflammation and proliferation stage. Thus, an imbalance in the micro-environment will alter the network of their functionality, which could lead to prolonged healing or excess scar formation[11]. The expression profile shows that monocyte chemoattractant protein-1 (MCP-1) production by keratinocytes is significantly high in normal wound healing[12]. However, an *in vivo* study revealed that the reepithelization is delayed in MCP-1 deficient mice, indicating that the

dysfunction of the chemokine-dependent pathway could impair tissue remodeling[13]. Since chemokines are small proteins that do not have major modification regions other than two disulphide bonds, they are highly stable and can be used as adjuvants corresponding to their functional groups for wound therapy. Also, the ability of chemokines to bind G-protein-coupled receptors increases their likelihood as therapeutic targets for regulating biological activity, thereby mitigating disease progression[14]. This review examines chemokines/their specific receptors as potential targets for treating DFU and emphasizes the possible regulation to attain with HBOT for a combinatorial therapeutic approach to hasten the healing process.

Oxygen in wound healing

Oxygen is essential for maintaining basic cellular functions such as ATP production, protein synthesis, and reactive oxygen species (ROS) formation. ROS are oxygen molecules in a reduced format that are highly reactive. These radical derivatives are not only involved in the oxidative killing of bacteria but also act as secondary signal molecules[15]. Most well-known ROS molecules such as $\cdot O_2^-$, $\cdot O_2^{-2}$, H_2O_2, $\cdot OH$, and $\cdot OH^-$ are produced during oxidative phosphorylation. Like ROS, the reactive nitrogen species (RNS) are normal physiological by-products based on nitrogen oxidation that react mainly with thiols and transition metals to form nitrosyl-metal complexes. Cells such as macrophages, platelets, keratinocytes, and macrophages utilize these radical complexes as a signal response during wound healing[16]. However, their respective role in the cell cycle, homeostasis, cell-mediated defense, and activation of pro-apoptotic proteins for cell death significantly differs based on low, basal, high, and excessive concentrations. *In vivo* studies have shown that an optimal and sustained level of ROS mediates the secretion of pro-inflammatory cytokines and induces the matrix of metalloproteases. On the contrary, the addition of excessive reactive species (either ROS or RNS) was found to damage the ECM and diminish the function of dermal fibroblasts and keratinocytes. This shows that the lower and higher level of radical species has respective accelerating and decelerating effects on wound repair[17]. Thus, maintaining a balance in the level of oxidative species is critical for bringing effective outcomes.

Hyperglycaemia and oxidative stress

Diabetes is known to be accompanied by increased cellular oxidative stress. However, recent studies have only shown that hyperglycaemia resulting from unregulated blood glucose levels causes dysfunction in the antioxidant defense system by triggering the overproduction of ROS[18]. The hyperglycaemia-induced cellular damage is mainly associated with: (1) Excessive formation of advanced glycation end products; (2) Protein kinase C activation; and (3) Increased polyol and hexosamine pathway flux, all of which could enhance oxidative stress[19]. The exact mechanism is ambiguous as one influences the other. Still, the prevailing evidence suggests that hyperglycaemia increases the availability of electron transfer donors such as $FADH_2$ and NADH, which increases the electron flux, further creating hyperpolarization of mitochondrial membrane potential due to a change in ATP/ADP ratio. The high electrochemical potential difference leads to the partial inhibition of electron transport between coenzyme Q and complex III, resulting in electron accumulation, further driving the partially reduced O_2 to generate free radical anion superoxide, and thus impairing the cell function[20].

Circulatory endothelial progenitor cells (EPCs) produced in the bone marrow play a significant role in the regeneration of the endothelial lining of blood vessels in response to ischemic conditions[21]. The antioxidant enzyme level of EPCs was found to be enhanced in a low oxygen environment to engraft vasculogenesis. However, their activation and proliferation are significantly impaired in an oxidative stress environment and the baseline pattern is similar to that of diabetic conditions[22]. Other than activation and proliferation, the migration of EPCs to the injured sites followed by tissue homing based on chemokine signal is important for wound repair. Nevertheless, the process is diminished in diabetic condition due to signal deficit, further impairing EPC function[23]. Thus, the elevated level of ROS production is believed to be the prime factor by which hyperglycaemia-mediated diabetes affects the normal wound healing process.

HBOT and oxidative response in diabetic wound healing

The pathological state of delayed wound healing is associated with prolonged oxygen deficit. The increase in the amount of oxygen would generate a favorable gradient for its diffusion into the affected tissues[3]. The management of chronic non-healing wounds by HBOT increases the rate of oxygen perfusion 10-50 folds and shows a correlation by modulating the inflammatory response with an increase in ROS production[24]. Vascular endothelial growth factor (VEGF), a key angiogenic factor responsible for maintaining blood vessel integrity, is stable under hyperoxia and hypoxia conditions. The function of endothelial-1, an endogenous vasoconstrictor responsible for the maintenance of blood pressure and basal vascular tone, seems to have significantly decreased under hyperoxic conditions[25]. Thus, it is paradoxical to perceive that the increase and decrease in oxygen concentration have alternative effects on blood vessels together with varying levels of ROS production, thus imparting a setback on HBOT.

A systematic study based on 9000 previous records on the effect of hyperoxygenation shows that HBOT increases the level of oxygen radicals and increases the chance of inducing oxidative stress. On the contrary, the meta-analysis data reveals that HBOT stimulates the release of angiogenesis-promoting cytokines and growth factors, whose function is impaired when oxidative stress is high, as in the case of diabetes[26]. The most remarkable understanding is obtained from the thermal imaging data of an HBOT-treated wound with decreased temperature, indicating a decline in inflammation[27]. Since no significant difference in the profile of anti-inflammatory markers was observed in HBOT, its direct role in anti-inflammation seems less probable. Thus, promoting a wound to an anti-inflammatory state from a prolonged inflammatory condition (where ROS level is high) could be possible by regulating a nuclear factor that suppresses the pro-inflammatory genes. Nuclear factor kappa B (NF-κB) is a critical transcriptional factor in inflammation that activates several pro-angiogenic genes together with HIF-1α under hypoxia conditions. However, inhibitor of kappa B alpha (IκBα), another nuclear factor that is stimulated under hyperoxic conditions, inhibits NF-κB, resulting in the downregulation of pro-inflammatory transcription factors and pushing the cellular environment towards an anti-inflammatory state [28]. HBOT could establish the same condition in the wound micro-environment despite oxidative stress and aids healing. An *in vivo* study validates that hyperoxia induced during HBOT session is associated with decreased NF-κB expression and stimulated the activation of IκBα, which is generally degraded under hypoxia[29]. Although it seems promising, no significant evidence is available about the cellular damage induced by the preformed oxidative species or its reversal effect by HBOT before the establishment of anti-inflammatory phase, which needs to be addressed through research for regulating the interventional procedure.

Macrophage polarization in normal vs diabetic wound

Macrophage infiltration on the wound site is mainly derived from the monocyte precursors in response to pathogen-associated modifying proteins or damage-associated modifying proteins. Besides the scavenging activity, macrophages play other roles in tissue regeneration and wound repair[30]. However, depending on the phenotype, their functionality is assigned in the tissue micro-environment.

The macrophages are divided into three subgroups based on the markers that they express on the surface: Classical macrophages ($CD14^{++}CD16^{-}$), intermediate macrophages ($CD14^{++}CD16^{+}$), and non-classical macrophages ($CD14^{++}CD16^{++}$)[31]. Classical macrophages are known as M1 or pro-inflammatory macrophages that are triggered by tumor necrosis factor (TNF) and lipopolysaccharides and produce pro-inflammatory cytokines such as interleukin (IL)-12 and IL-23, together with ROS. The non-classical macrophages are known as M2 or resolving macrophages that are stimulated by the anti-inflammatory cytokines such as IL-4 and IL-10 to activate the release of growth factors such as transforming growth factors and insulin-like growth factors[32]. In normal wound healing, M1 predominates up to 3 d in clearing up the pathogens and dead/dying cells from the wound site and causes inflammation. The transition to M2 occurs thereafter with a peak in activity on day 7, promoting wound healing. Studies have shown that impairment in the transition to M2 and persistent polarization of M1 macrophages are responsible for prolonged wound healing in chronic disease conditions[31]. An *in vivo* study showed that the ratio of CCR7-CD48, a respective chemokine receptor and marker found on M1 and M2 macrophages, is higher in diabetic mice with impaired wound healing than in controls, indicating the dysfunctionality of macrophage switching/transition and its importance in wound repair. Also, it has been shown that the prolonged M1 polarization reduces the expression of matrix metalloproteinases together with increased secretion of pro-inflammatory cytokines such as TNF-α, affecting the keratinocyte migration and leading to the concussion of normal wound healing process[33]. Several factors were found to contribute to the persistent polarization of M1 macrophages, which is why the therapeutic development could be focused on either blocking the inflammatory cascade activating the M1 phenotype or promoting M2 transition to resolve the debilitating diabetic chronic wounds.

Chemokines - a potential regulator of macrophage polarization and wound differentiation

Chemokines are a family of secretory proteins with low molecular weight (8-12 kDa) that have a prominent role in chemotaxis and activation of immune cells. The four subfamilies of chemokines C, CC, CXC, and CX3C are classified based on the two conserved cysteine residues present at the N-terminal motif[34]. Chemokines are important in regulating angiogenesis during hemostasis and the inflammatory phase of wound healing for clot formation and the influx and efflux of migratory cells. Also, they control the formation and regression of neovessels during the proliferation and remodeling phase to assist the healing wound in meeting the metabolic need and scar formation. Thus, playing a pivotal role in orchestrating the precise sequence of events, chemokines are crucial in all stages of wound healing (Table 1)[35]. As discussed earlier, for the establishment of the proliferation phase, the pre-formed inflammation in the tissue microenvironment should get declined by the anti-inflammatory signal cascade to establish the transition of the M1 to M2 phenotype to aid tissue repair.

Adipose tissue macrophages constitute 10%-15% of the total cell population in healthy individuals, and they predominantly show M2 phenotype with high insulin sensitivity. However, in obesity, the adipocytes secrete pro-inflammatory markers that trigger the recruitment of monocytes *via* the CCL5-CCR5 pathway. The macrophages derived from those monocyte precursors acquire the M1 phenotype and contribute to prolonged inflammatory environments[36]. On the contrary, the intrahepatic

Table 1 Notable chemokines and their function at different stages of wound healing with their relative expression in normal *vs* diabetic condition

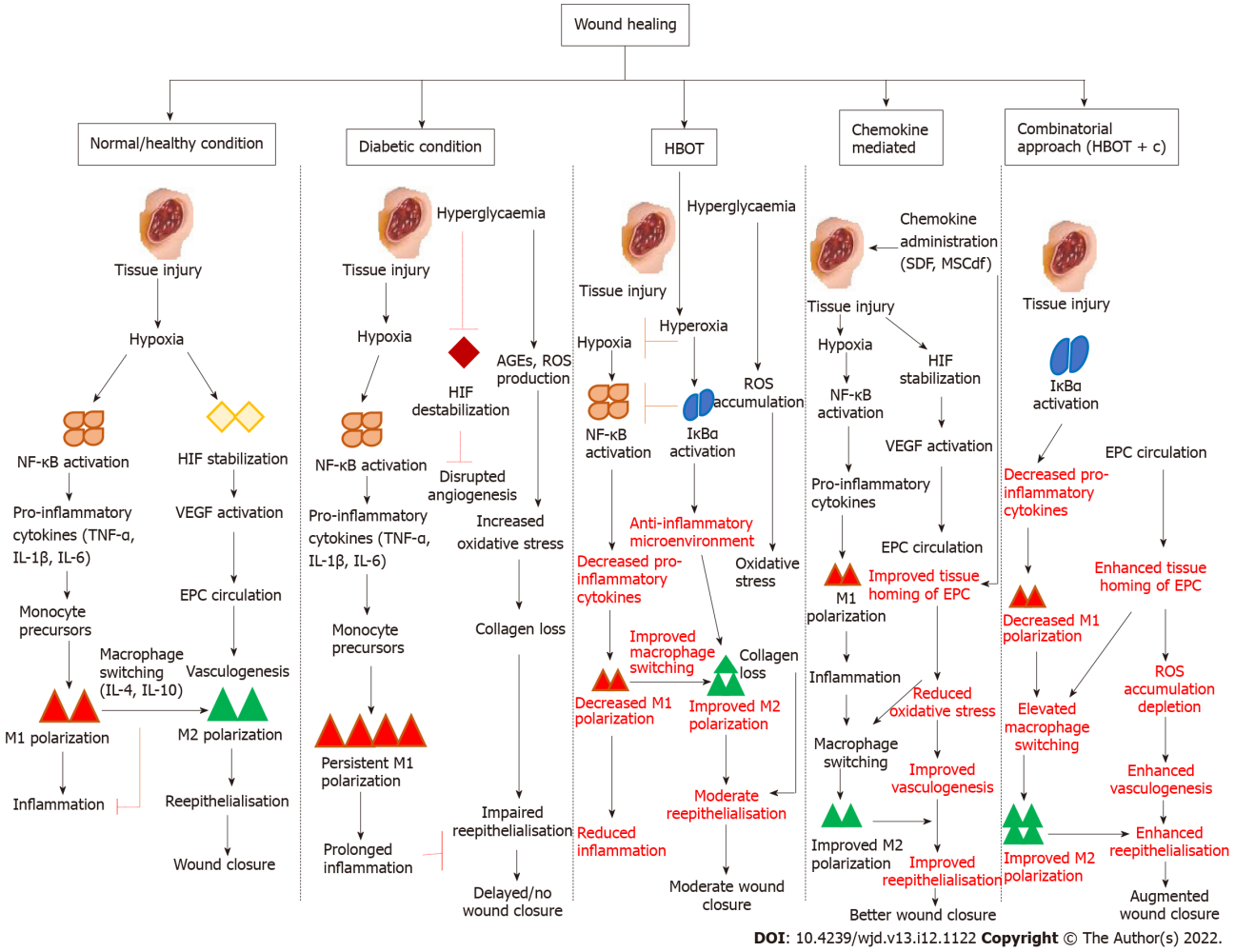
Chemokine	Stage	Function	Role	Relative expression in normal wound	Relative expression in diabetic wound
CXCL4/platelet factor 4	Hemostasis	Angiostatic	Inhibition of VEGF-induced VEC proliferation	+++	+
CCL2	Inflammation	Macrophage recruitment	Activation of P38MAPK pathway	+++	++++
CCL5	Inflammation	Eosinophil recruitment	PKB phosphorylation to induce apoptosis	+++	++
CXCL8	Hemostasis & inflammation	Neutrophil recruitment	Phagocytosis	+++	+
CCL3	Proliferation	Macrophage polarization	ECM formation	+++	Unknown
CXCL11	Proliferation	Angiostatic	Basement membrane regeneration	+	Unknown
CXCL12	Proliferation	Granulation tissue formation	Unknown	++	NA
CCL2	Proliferation	Type-I collagen deposition	Upregulation of MMP-1	+++	NA

+: Denotes minor increase in expression; ++: Denotes moderate increase in expression; +++: Denotes large increase in expression; NA: Not available; CXCL: chemokine (C-X-C motif) ligand; CCL: Chemokine ligands; VEGF: Vascular endothelial growth factor; VEC: Vascular endothelial cells; MAPK: Mitogen activated protein kinase; PKB: Protein kinase B; ECM: Extra cellular matrix; MMP: Matrix metallopeptidase.

monocytes in the presence of the anti-apoptotic protein BCL2 are mediated by the CX3CL1-CX3CR1 pathway and show a less inflammatory phenotype characterized by decreased TNF-α and nitric oxide synthase production[37]. Thus, macrophage polarization in metabolic disorders could be modulated by regulating the chemokines and their specific receptors.

Recent advancement in stem cell-based approaches has garnered significant interest as they have potential therapeutic value. Studies have shown that exosomes derived from mesenchymal stem cells (MSCs) possess immunomodulatory effects that can induce the transition of pro-inflammatory macrophages to anti-inflammatory phenotype in various inflammatory-associated disease conditions [38]. These MSC-derived exosomes exhibit high expression of angiogenic and tissue repair factors such as VEGF, extracellular matrix metalloproteinases, and matrix metallopeptidase 9[39]. The adipocyte-derived MSCs that express Arg-1 and IL-10 based on the activation of STAT3 transferred by the exosomes are shown to alleviate inflammation through M2 polarization[40]. The genes present downstream of macrophage transcriptional factors, such as interferon regulatory factor/STAT, which arranges the polarization. Bruton's tyrosine kinase with STAT1/STAT5 and Kruppel-like factor 4 with STAT3/STAT6 induce the polarization of the M1 and M2 phenotype, respectively[41,42]. However, for the complete induction of the anti-inflammatory phenotype, the peroxisome proliferator-activated receptor gamma is essential, and its absence diminishes insulin sensitivity, further resulting in hyperglycaemia that impairs cellular function[43].

Other than MSCs, epidermal stem cells aid in tissue repair by modulating the migration and proliferation of EPCs to the injury site. Abnormal EPC migration and a lack of tubularization cause impaired angiogenesis in diabetic patients[21]. Stromal cell-derived factor 1 (SDF-1), a chemokine belonging to the CXC family, recruits EPC to the wound site by interacting with CXCR receptors 4 and 7. A study has shown that the expression of SDF-1 between acute and chronic wounds differs significantly. In the case of chronic wounds, no influence was observed with EPC migration, thus, an exogenous administration of SDF-1 is inevitable to accelerate the wound healing rate[44]. However, the duration of the chemokine gradient and its bioavailability are essential factors to consider in the effectiveness of the wound-healing rate. Instead of a single dose administration, a formulation that enhances a slow release of the element might have a significant positive effect on tissue regeneration. To achieve this, a biomaterial scaffold that retains the bioactivity of chemokine could be developed for tissue engineering purposes. SDF-1 encapsulated in poly(ethylene glycol citrate-*co*-N-isopropyl acrylamide) has improved the tissue healing rate in diabetic mice with sustained release of the factor for up to 3 wk without any burst[45]. Modifying the hydrogel systems, such as integrating anti-oxidant properties, could render more advantages for rapid healing, and developing such state-of-the-art techniques could revolutionize the therapeutic aspects of treating chronic diabetic wounds.



DOI: 10.4239/wjd.v13.i12.1122

Figure 1 Mechanism of wound healing in normal *vs* diabetic conditions and plausible molecular regulations achieved by hyperbaric oxygen therapy, chemokine administration, and combinatorial approach (hyperbaric oxygen therapy + chemokine administration) for augmented therapy. HBOT: Hyperbaric oxygen therapy; SDF: Stromal cell-derived factor; MSC: Mesenchymal stem cell; AGEs: Advanced glycation end products; ROS: Reactive oxygen species; HIF: Hypoxia-inducible factor; VEGF: Vascular endothelial growth factor; EPC: Endothelial progenitor cell; NF-κB: Nuclear factor kappa B; TNF: Tumor-necrosis factor; IL: Interleukin; IκBα: Inhibitor of kappa B alpha.

CONCLUSION

Diabetes is a chronic disease that brings delirious effects through prolonged inflammation that could lead to other metabolic disorders such as cardiovascular diseases, hypertension, and renal diseases. Several interventions have been suggested, including a healthy diet, exercise, and proper medication to lessen the adverse outcomes. However, a better therapeutic approach is needed for an effective outcome despite the standard procedures. The problem with delayed wound healing and persistent infection in diabetic patients is attributed to the deficiency of oxygen perfusion to the injured site. The resulting hypoxic environment alters the sequence of cellular events from the normal wound healing and complicates the process further. HBOT is found to fasten the wound healing rate in DFU cases by inducing angiogenic factors and other critical components of the cellular cascade. Although HBOT is found to be efficient in reverting the ischemic condition of the wound, complete reliance on the interventional procedure is not enough, as wound healing is a multifactorial process. Thus, the efficiency of chemokine-mediated response is essential for activating the effector cells that participate in wound healing.

The combinatorial therapeutic approach could be of interest as it will likely lead to a better outcome. HBOT and simultaneous administration of tissue-specific chemokine/receptor modulating factors could overcome multiple wound healing deficits observed in diabetic conditions (Figure 1). Since not much research was carried out earlier with the proposed combination, this review emphasizes the researchers to conduct various controlled trial studies with Food and Drug Administration-approved biologics to explore the potential and developing novel strategies and better clinical practices for treating diabetic wounds.

ACKNOWLEDGEMENTS

The authors would like to thank Vellore Institute of Technology for providing necessary resources for preparing this review.

FOOTNOTES

Author contributions: Jaganathan V and Pragasam V designed and wrote the manuscript; and all authors have read and approved the final manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Wang JJ

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1131-1139

DOI: 10.4239/wjd.v13.i12.1131

ISSN 1948-9358 (online)

MINIREVIEWS

The role of artificial intelligence technology in the care of diabetic foot ulcers: the past, the present, and the future

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Mijwil MM, Iraq; Mostafavinia A, Iran; Wu QN, China

Received: August 25, 2022
Peer-review started: August 25, 2022
First decision: October 30, 2022
Revised: November 1, 2022
Accepted: December 1, 2022
Article in press: December 1, 2022
Published online: December 15, 2022

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Abstract

Foot ulcers are common complications of diabetes mellitus and substantially increase the morbidity and mortality due to this disease. Wound care by regular monitoring of the progress of healing with clinical review of the ulcers, dressing changes, appropriate antibiotic therapy for infection and proper offloading of the ulcer are the cornerstones of the management of foot ulcers. Assessing the progress of foot ulcers can be a challenge for the clinician and patient due to logistic issues such as regular attendance in the clinic. Foot clinics are often busy and because of manpower issues, ulcer reviews can be delayed with detrimental effects on the healing as a result of a lack of appropriate and timely changes in management. Wound photographs have been historically useful to assess the progress of diabetic foot ulcers over the past few decades. Mobile phones with digital cameras have recently revolutionized the capture of foot ulcer images. Patients can send ulcer photographs to diabetes care professionals electronically for remote monitoring, largely avoiding the logistics of patient transport to clinics with a reduction on clinic pressures. Artificial intelligence-based technologies have been developed in recent years to improve this remote monitoring of diabetic foot ulcers with the use of mobile apps. This is expected to make a huge impact on diabetic foot ulcer care with further research and development of more accurate and scientific technologies in future. This clinical update review aims to compile evidence on this hot topic to empower clinicians with the latest developments in the field.

Key Words: Diabetic foot ulcers; Photographic monitoring; Artificial intelligence technology; Digital photography; Mobile app; COVID-19 pandemic

Core Tip: Diabetic foot clinics faced major challenges during the COVID-19 pandemic due to lockdowns and social distancing measures as a significant proportion of patients were unable to physically attend the clinics. This situation boosted the attempts for transition of face-to-face foot clinics to virtual clinics as in many other types of medical care during the pandemic. Monitoring of diabetic foot ulcers (DFUs) by digital photographic technology and mobile phone-based photography have revolutionized this area of clinical care in recent years and mobile apps are expected to accelerate this progress. This article reviews the past, the present and the future of artificial intelligence technology in the care of DFUs.

Citation: Pappachan JM, Cassidy B, Fernandez CJ, Chandrabalan V, Yap MH. The role of artificial intelligence technology in the care of diabetic foot ulcers: the past, the present, and the future. *World J Diabetes* 2022; 13(12): 1131-1139
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1131.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1131

INTRODUCTION

Foot ulceration caused by micro- and/or macrovascular disease is a common complication of diabetes mellitus (DM). The global prevalence of diabetic foot ulcers (DFUs) at any point in time is estimated to be 6.3%[1], with 25% of DM patients developing a DFU in their lifetime[2]. DFUs significantly increase morbidity [including lower extremity amputations (LEA)] and mortality among sufferers, and the care of DFUs poses very high healthcare expenditure worldwide. Approximately 50% of DFUs are associated with infections[3], and approximately 20% of moderate to severe DFU infections may lead to minor or major amputations[4]. DFU-related LEA is associated with a 10-year survival rate as low as 24% which is lower than that of several forms of cancers[5]. These alarming figures give us a broad outline of the health-related risks posed by DFUs.

Appropriate care of DFUs to ensure rapid cure and avoid complications involves regular monitoring of the progress of healing with periodic review of the ulcers, frequent dressing changes, antibiotic therapy for infection control, optimal control of DM, and adequate offloading of the ulcers to avoid ongoing damage due to pressure on the wound area. Diabetic foot clinics usually provide comprehensive DFU care by multidisciplinary specialist teams involving podiatrists, orthotists (who make appropriate footwear/devices for offloading the DFU), diabetes specialist nurses, and diabetologists. However, insufficient manpower, long clinic waiting lists, and logistic issues with patient transport to the clinics can all pose problems for timely review of patients with DFUs in the foot clinics. Manpower shortage resulting in longer waiting periods prior to review by the footcare team has been a major issue in ulcer care in diabetes foot services across the world in recent years[6,7]. Delays in presentation for foot ulcer review have been identified as an important reason for wound non-healing[8]. Complications of foot ulcers including amputations can be the devastating sequel of such delays in review by the footcare team.

The COVID-19 pandemic posed major challenges in DFU care across the globe due the above reasons [9-11]. Delays in ulcer reviews may be associated with detrimental effects on DFU healing that may even lead to amputations[10,11]. Telephone and video clinics during the pandemic period helped clinicians to address some of the issues related to the inability of patients to attend medical clinics during the COVID-19 lock-down period. Attempts to develop artificial intelligence (AI) algorithms by the scientific fraternity to enhance patient care through virtual clinics attained greater momentum in relation to the pandemic[12,13].

Monitoring the progress of DFUs by comparing serial ulcer photographs during the follow-up in foot clinics has been practiced by many diabetologists over the past 2-3 decades. Refinement of the photographic methods by sophisticated digital technology and mobile phone cameras have revolutionized DFU care in recent years[14-16]. Some patients often use mobile phone cameras to capture their foot ulcer photographs for self-monitoring wound progress and to help clinicians to understand the previous status of their wound during clinic review.

However, much more multidisciplinary scientific research input and output are necessary in this area. Further refinement of this rapidly advancing digital technology for optimal use in day-to-day clinical practice could result in better care of patients with DFUs. This review attempts to gather up-to-

date evidence to summarise the past, present, and future dimensions of AI algorithms to empower clinicians across the globe to appropriately utilise digital technology for DFU care.

PHOTOGRAPHIC MONITORING OF DFU PROGRESS

Diabetes care professionals often see several cases of DFU in their day-to-day clinical practice and often forget the previous ulcer grade, character and even the site during subsequent visits, weeks later when followed up in the foot clinic. Review of the previous photographs during subsequent follow-up visits should provide clinicians with a good clinical assessment of the progress of the ulcer and help prognostication[17]. Photographic monitoring also enhances appropriate continuity of care by different clinicians running the diabetic foot services during their review of the same case.

Important issues which can arise while comparing the photographs are the differences in the distance from which photographs are captured (can be largely avoided by placing a measuring tape on the ulcer while photographed), differences in the illumination of pictures from brightness of background light when the photographs were taken, and the likelihood of low-quality images without adequate focusing of cameras by individuals taking the picture. Photographs are usually taken without flashlight to avoid undue illumination that can reduce the picture quality. Clinical evidence suggests that prediction of ulcer healing is possible by regular photographic monitoring[17]. Figure 1 shows the photographic monitoring of the progress of a DFU at various stages.

HISTORY OF DIGITAL ARCHIVING OF THE DFU PHOTOGRAPHS

Use of digital cameras for photography has become a common practice since the early 1980s after the invention of digital photographic technology in 1975. Digital archiving of foot ulcer photographs in computer databases was a great leap forward in the technology for monitoring DFUs. Print outs of foot ulcer photographs are still used in remote settings where internet and computers are not freely available in clinical practice. This is in fact more expensive (costs incurred with colour printing and use of good quality photographic paper) and cumbersome in the modern era.

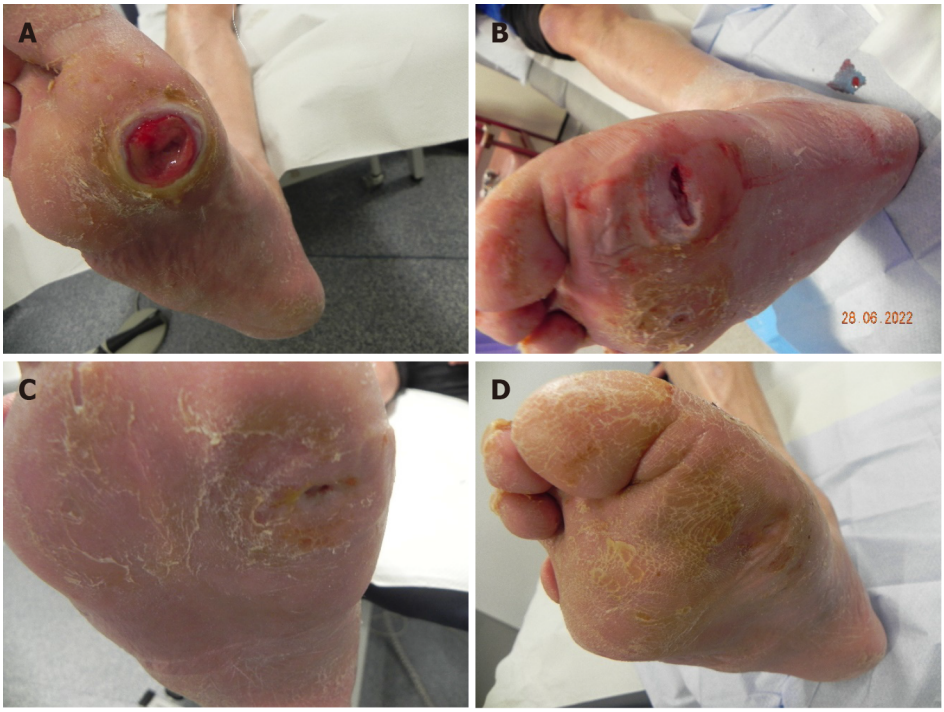
Fading of the colour print outs over time makes the situation worse regarding DFU monitoring using this method. A review of the serial images in the computer database of digital photographs makes the work of diabetes foot care professionals much easier when this facility is available[18]. Lack of degradation of image quality over time as in printed photographs is another great advantage of digital archiving of DFU images.

Mobile phones became popular for telephone communications globally in the early 2000s and newer versions of these devices with cameras and video recording facilities came into the market a few years later. With rapid advancements in technology, smartphone devices using high resolution cameras are now widely available and have become an integral part of modern life in the 21st century. Diabetes care professionals soon started using mobile phone cameras and video facilities for the monitoring and management of DFUs[19-21]. Some patients themselves were using mobile phones to obtain images of their DFUs prior to their foot clinic visits so that they could show the status of their ulcers earlier. Although the initial studies on use of mobile phones for monitoring DFUs were not very promising[22], subsequent studies show encouraging results[23-25].

INTEGRATING DFU PHOTOGRAPHS WITH COMPUTER-BASED DIGITAL TECHNOLOGY FOR DIAGNOSTICS

AI and its applications have been utilised in various branches of modern science and technology including the medical field over the past few decades to improve physical and intellectual human work output. Such technologies can also be utilised to monitor the progress in DFU care. DFU datasets were utilised for training and testing the machine learning processes. The collection of such datasets supports the ongoing research for DFU academic AI challenges, such as those hosted by the Medical Image Computing and Computer Assisted Interventions (MICCAI) conferences[26,27]. These challenges are used to promote and advance ongoing research and to increase exposure within the associated academic fields.

Machine learning algorithms have been found to be very useful in detecting DFUs with high accuracy rates in previous studies[28-30]. These algorithms are developed by using large datasets of images captured from the foot clinics. The development of computer-aided DFU diagnostic algorithms involves multiple stages such as pre-processing, feature extraction, detection, classification, and segmentation of DFU wounds[30]. These tasks can be challenging in real-world settings due to the low-quality of images from inadequate focusing, motion artifacts, inadequate lighting, and backlight, deformities in the foot/toes, size and shape of the ulcers (very small, very large and curved ulcers), and newly formed or



DOI: 10.4239/wjd.v13.i12.1131

Figure 1 Photographic monitoring of the progress of a DFU at various stages. A: Infected foot ulcer on April 25, 2022; B: After 2 mo of regular offloading and dressings (on June 28, 2022) with initial 3 wk of antibiotic therapy; C: Further improvement of ulcer on July 23, 2022: D: Complete healing of ulcer on August 22, 2022.

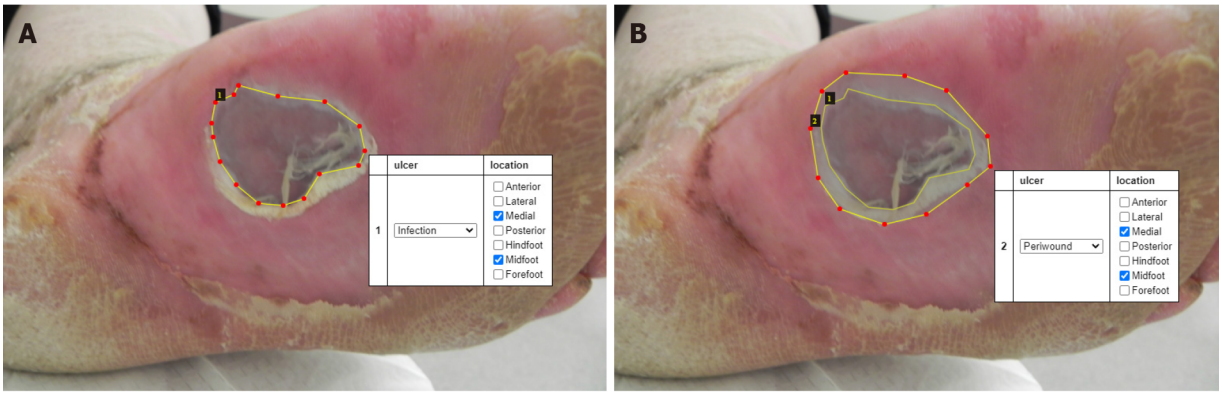
early ulcers which are easily missed during the capture of photographs in foot clinics[16].

The first and foremost step in the development of machine learning algorithms is the detection of DFUs from foot photographs. This task has been successfully carried out by previous researchers as mentioned above[28-30]. Further refinement of machine learning is currently being undertaken by incorporating the classification systems for DFUs, as in the real-world settings, to promote AI-based diagnostics and prognostication. Several manual DFU classification systems are currently used by foot care professionals such as Wagner, University of Texas, and SINBAD (Site, Ischaemia, Neuropathy, Bacterial infection, Area, and Depth) for DFU monitoring and management[30]. These manual approaches may benefit from the automated processes afforded by AI.

Incorporation of such complex features in the AI-technology for day-to-day clinical practice to enhance diagnosis and prognostication can be challenging. These challenges include: (1) The significant time-burden involved in the DFU image data collection and appropriate labelling; (2) The inter- and intra-class variations depending on differences in the classification of DFUs; (3) Lack of standardization of DFU datasets (caused by camera distance from the foot, image orientation and lighting conditions); and (4) The differences in ethnicity, age, sex, and foot size of the patients[30]. Development of deep learning AI algorithms requires large-scale datasets for automated DFU analysis to reproduce comparable results to those by experts. Researchers currently working in isolation may not achieve reproducible research outputs. Large DFU datasets used for training and validation by multiple professionals from different institutions across the globe should help to refine these pitfalls in machine learning algorithms for DFU classification and diagnosis. To enable innovation from clinicians and researchers, Yap *et al*[26] proposed the diabetic foot ulcer challenges by providing the publicly available datasets, for comprehensive evaluation of object detection frameworks on DFU detection using convolutional neural networks trained on the DFUC2020 dataset[15]. Examples of manually delineated DFU photographs from the training set are shown in Figure 2. Morphological classification of DFUs into different types (such as infection, ischaemia, both, and none) is carried out in these datasets to enable machine learning.

SERIAL DEMARCATION OF ULCER PHOTOGRAPHS TO ASSESS PROGRESS OF DFU HEALING

At present, monitoring the healing process of DFUs is largely completed by regular patient follow-up visits in the multidisciplinary foot clinics[31-33]. Since 2020, the COVID-19 pandemic has made a huge impact on DFU care across the world due to the issues related to lockdowns and social distancing



DOI: 10.4239/wjd.v13.i12.1131

Figure 2 An infected ulcer on the plantar aspect of the left foot. A: The ulcer is labelled (dotted line marking the boundaries) for the training set. The white box shows the site and type of ulcer. B: The peri-wound of the ulcer is labelled with a dotted line. The white box shows the site of the ulcer and the peri-wound.

measures enforced by governments to curtail human devastation as a result of the global health emergency. Foot clinic attendance[34] and hospitalisations for DFUs[35] were significantly reduced during the pandemic. Although worsening of DFUs was comparatively less owing to reduced outdoor human activity[36], many patients presented to clinicians late, increasing the risk of complications from DFUs[35,37]. The pandemic reinforced the urgent necessity to develop AI-based wound care algorithms for remote monitoring of DFUs.

To enable the development of machine learning algorithms, serial demarcation of DFUs is important to assess the progress of the healing process along with wound classification based on one of the above-mentioned standard systems. Therefore, characterisation of DFUs requires demarcation of wounds at various stages in the datasets by experts in the field after which the data can be used for training, validation, and testing. This task is highly labour-intensive and requires huge DFU training and validation datasets.

PHOTOGRAPHS ACQUIRED BY MOBILE PHONE CAMERAS FOR DFU MONITORING

New generation mobile phones can capture high resolution DFU photographs comparable to commercial digital cameras. Many patients already use mobile phone photography for self-monitoring their DFUs[14,22]. Some patients serially document their wound progress using these images and meticulously bring them to foot clinics during their follow-up visits for DFU care. Occasionally, patients send their photographs to footcare professionals electronically or as email attachments. Although safety issues related to secure transfer of such clinical information without breach of confidentiality can be challenging at present, wider use of mobile phone photography for self-monitoring of DFUs progress might revolutionise future foot care.

DEVELOPMENT OF MOBILE APPS FOR DFU MONITORING

Various mobile phone apps are currently used worldwide in many domains of daily activities to improve the quality of human life. Such mobile apps for DFU monitoring and care are under development. Cassidy *et al*[38] developed the first mobile app capable of accurate DFU detection using AI and cloud-based technologies. This system was tested in a 6-mo clinical evaluation at two UK National Health Service hospital sites (Lancashire Teaching Hospitals and Salford Royal Hospital) and is currently being further developed to improve functionality and accuracy. Additional app features, such as automated classification of DFU wound pathology[39] and automated delineation of wound/peri-wound regions are also being investigated to provide a more clinically relevant system.

These new technological advancements are expected to revolutionise remote wound care by enabling patients to self-monitor their DFUs and contact clinicians when they find any concern or deterioration of their disease. This would also enhance flexibility in the functioning of foot clinics by reviewing and triaging the most appropriate DFUs to be seen in the clinics by remote monitoring of patients.

PREDICTION OF DFU PROGRESS BY INTEGRATION OF CLINICAL AND BIOCHEMICAL PARAMETERS AND ULCER PHOTOGRAPHS

Although regular wound care with dressings, appropriate antibiotic therapy for infections, offloading the ulcers to relieve pressure-related delay in healing and revascularisation of the ischaemic foot are the cornerstones in the management of DFUs, several other clinical (comorbid conditions such as renal disease, heart failure, and immunosuppressed states) and biochemical parameters (such as hyperglycaemia, anaemia, and high haemoglobin A1c) may impact the DFU healing process[40-44]. Integration of these clinical and biochemical parameters into machine learning algorithms should help us to develop prediction models using AI technology.

We note however that the development of such algorithms is much more labour-intensive as demarcation exercises to develop deep learning models require larger datasets with clinical and biochemical parameters of individual patients incorporated within the neural network. However, appropriate use of computer technology integrated with digital applications can help to reduce the physical burden on researchers in developing such models.

FUTURE PERSPECTIVES

Recent research has investigated the utilisation of patient data in the training of deep neural networks in various medical imaging domains[45]. These studies indicate that machine learning models trained on patient data can be used to boost the performance of convolutional neural networks trained on wound/lesion images. Research of this nature is ongoing and represents a way to incorporate a more integrated method of wound analysis that considers multiple data points. The development of mobile apps integrating such new advancements in technology is expected to revolutionise the global scenario of DFU care in the near future.

CONCLUSION

Digital applications in the daily management of DFUs have evolved rapidly in recent years to a level of remote diagnosis and monitoring of wounds in community settings. The COVID-19 pandemic has accelerated research and development of such innovative technological applications in the past two years. Photographic monitoring of foot ulcers has been practiced in many centres across the world in the past few decades providing DFU care. The invention of digital photographic technology in 1975 further boosted DFU care because of the ease of electronic archiving of ulcer images during clinical follow-up. Photography using mobile phone cameras has become a huge leap forward in this direction in recent years empowering patients and clinicians to further improve DFU care.

AI-based digital algorithms are currently being developed rapidly through collaborative global effort between AI experts and clinical teams. Mobile camera-based digital technologic applications are under development to enhance remote diagnosis, monitoring, and follow-up care of DFUs. Prediction models of wound healing are also under development now making use of linking the ulcer characteristics of DFU images to the clinical and laboratory parameters of diabetic patients. These collaborative efforts between clinicians and computer scientists across the world should revolutionise such discoveries to empower diabetic foot patients to self-monitor and manage their DFUs to a greater extent.

FOOTNOTES

Author contributions: Pappachan JP substantially contributed to the conception and design of the article, interpretation of relevant literature, article drafting, and figure preparation; Cassidy B contributed to the interpretation of relevant literature, article drafting, and figure preparation; Fernandez CJ and Chandrabalan V contributed to the literature search and revision of the article; Yap MH supervised article preparation and revised the manuscript critically for important intellectual content; All authors have read and approved the final version of the manuscript.

Conflict-of-interest statement: All authors report no relevant conflict of interest for this article.

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S-Editor: Liu GL
L-Editor: Webster JR
P-Editor: Liu GL

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1140-1153

DOI: 10.4239/wjd.v13.i12.1140

ISSN 1948-9358 (online)

MINIREVIEWS

Single nucleotide variations in the development of diabetic foot ulcer: A narrative review

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Specialty type: Endocrinology and metabolism

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Mrozikiewicz-Rakowska B, Poland; Primadhi RA, Indonesia

Received: August 27, 2022
Peer-review started: August 27, 2022
First decision: October 30, 2022
Revised: November 24, 2022
Accepted: December 5, 2022
Article in press: December 5, 2022
Published online: December 15, 2022

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Abstract

Diabetes mellitus has become a global health problem, and the number of patients with diabetic foot ulcers (DFU) is rapidly increasing. Currently, DFU still poses great challenges to physicians, as the treatment is complex, with high risks of infection, recurrence, limb amputation, and even death. Therefore, a comprehensive understanding of DFU pathogenesis is of great importance. In this review, we summarized recent findings regarding the DFU development from the perspective of single-nucleotide variations (SNVs). Studies have shown that SNVs located in the genes encoding C-reactive protein, interleukin-6, tumor necrosis factor-alpha, stromal cell-derived factor-1, vascular endothelial growth factor, nuclear factor erythroid-2-related factor 2, sirtuin 1, intercellular adhesion molecule 1, monocyte chemoattractant protein-1, endothelial nitric oxide synthase, heat shock protein 70, hypoxia inducible factor 1 alpha, lysyl oxidase, intelectin 1, mitogen-activated protein kinase 14, toll-like receptors, osteoprotegerin, vitamin D receptor, and fibrinogen may be associated with the development of DFU. However, considering the limitations of the present investigations, future multi-center studies with larger sample sizes, as well as in-depth mechanistic research are warranted.

Key Words: Diabetic foot; Diabetic foot ulcer; Diabetic foot osteomyelitis; Single nucleotide variations; Narrative review

Core Tip: The pathogenesis of diabetic foot ulcer (DFU) is complex and is associated with both extrinsic and intrinsic factors. Most previous studies have reported the roles of external factors in DFU development and have neglected internal factors. In this narrative review, we focused on single-nucleotide variations (SNVs), as a representative of host factors. We summarized recent findings regarding the relationships between genetic SNVs and susceptibility of different populations to DFU. Future multicenter investigations with larger sample sizes, as well as in-depth mechanistic research, are necessary to better recognize and understand the roles of SNVs in DFU pathogenesis.

Citation: Hu YJ, Song CS, Jiang N. Single nucleotide variations in the development of diabetic foot ulcer: A narrative review. *World J Diabetes* 2022; 13(12): 1140-1153
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1140.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1140

INTRODUCTION

Diabetes mellitus (DM), one of the most frequently encountered metabolic disorders, has become a global health problem and is considered a public health emergency[1]. The severity of DM is not only attributed to the disorder itself but also to its associated complications, influencing both life expectancy and quality of life[2]. DM-related complications affecting the lower extremities are common, complex, and costly ("3Cs"), with diabetic foot ulcer (DFU) being the most frequently recognized type[3]. It is estimated that the lifetime incidence of DF or DFU is approximately 15%–25% among patients with DM [4,5]. DFU remains one of the most challenging disorders for physicians to treat, with a high risk of infection, recurrence leading to limb amputation, and even death. Over half of DFUs are infected[6]; the incidence of DFU recurrence is 40% within 1 year and 65% within 3 years[3]. Despite various treatment strategies, approximately 20% of DFU patients with moderate and severe infections experience different levels of amputation[7,8]. According to a database analysis from the United Kingdom, the risk of death at 5 years for DFU patients was 2.5-fold greater than that for DM patients without DFU[9]. Additionally, treatment of DFU is costly, with nearly one-third of the estimated expenses for DM spent on DFU[10-12].

The great hazards of DFU necessitate a comprehensive understanding of its pathogenesis, aiming at increasing the cure rate, and decreasing the risks of infection, recurrence, and death. The progression of DFU is complex, with diabetic neuropathy (DN) and peripheral artery disease being the primary causes [13]. Multiple factors participate in the development of DFU; however, most previous studies have focused on environmental and controllable host factors. Recently, growing evidence has revealed that as a representative of host factors, single-nucleotide variations (SNVs) or single nucleotide polymorphisms are also involved in the development of DFU. This narrative review summarized current investigations regarding the roles of SNVs in the occurrence of DFU, thus providing new insights into the pathogenesis of DFU.

GENETIC SNVS INVOLVED IN DFU DEVELOPMENT

C-reactive protein

As an acute-phase response protein, C-reactive protein (CRP) levels increase in cases of tissue injury, infection, inflammation, and cancer[14,15]. Furthermore, it can be up to 1000 times the normal value in severe situations. A recent meta-analysis[16] indicated that the role of CRP is a promising biomarker for DFU infection evaluation. The CRP protein, encoded by the *CRP* gene, is located on chromosome 1q21-q23 and is 2.3 kb long[17]. Recent studies have reported that *CRP* genetic SNVs associated with the risk of developing DFU, including rs11265260, rs1800947, rs2794520, rs1130864, and rs3093059 (Table 1).

In a 2020 case-control study, Wang *et al*[17] investigated the potential influence of CRP SNVs, together with environmental factors, on the development of diabetic foot osteomyelitis (DFO) and prognosis of the patients with DFO. Altogether, 681 patients with DFO, 1053 patients without DFO, and 1261 healthy controls were included; and 11 CRP SNVs were analyzed. The results showed that rs11265260 (allele G), rs1800947 (allele G), rs2794520 (allele T), and rs1130864 (allele T) were linked to an increased risk to develop DFO in this Chinese cohort. Additionally, rs3093059 (allele C) showed a decreased risk. Furthermore, rs11265260 (allele G), rs1800947 (allele G), rs3093068 (allele G), and rs1130864 (allele T) were significant predictors of poor prognosis in these patients. Moreover, the GG and AG genotypes of rs11265260, the CG and GG genotypes of rs1800947, the TT genotype of rs3093059, and the CT and TT genotypes of rs113084 amplified the influences of smoking, alcohol consumption, cacosmia, and ulceration on progression from non-DFO to DFO. These outcomes imply that both

Table 1 Single nucleotide variations involving in the development of diabetic foot and its related complications

Ref.	Population or ethnicity	Total sample size (DF *vs* T2DM without DF) *vs* controls	Genes	SNVs reported	Potential influences of the SNVs on DF and DF related complications	Genotypes as risk or protective factors
Wang *et al*[17], 2020	Chinese	2995 (681 *vs* 1053 *vs* 1261)	*CRP*	rs11265260	Risk factor of DFO	GG + AG/GG
			CRP	rs1800947	Risk factor of DFO	GG + CG
			CRP	rs2794520	Risk factor of DFO	TT + CT/TT
			CRP	rs1130864	Risk factor of DFO	TT + CT/TT
			CRP	rs3093059	Protective factor against DFO	CC+CT/CC
Dhamodharan *et al*[23], 2015	Indian	515 (270[1] *vs* 139 *vs* 106)	*IL-6*	rs1800795	Protective factor against T2DM but not against DFU-DN	GC, CC
Erdogan *et al*[24], 2017	Turkish	204 (50 *vs* 35 *vs* 119)	*IL-6*	rs1800795	Risk factor of T2DM but not DFU	GG
Viswanathan *et al*[25], 2018	Indian	270 (without controls)	*IL-6*	rs1800795	Risk factor of severe wound infections	GC + CC
Dhamodharan *et al*[23], 2015	Indian	515 (270[1] *vs* 139 *vs* 106)	*TNF-α*	rs1800629	Risk factor of both T2DM and DFU-DN	GA, AA
Viswanathan *et al*[25], 2018	Indian	270 (without controls)	*TNF-α*	rs1800629	Risk factors of severe wound infections, ulcer grade of DF	GA + AA
				rs361525	Risk factor of ulcer grade of DF	GA + AA
Dhamodharan *et al*[23], 2015	Indian	515 (270[1] *vs* 139 *vs* 106)	*SDF-1*	rs1801157	Protective factor against T2DM and/or DFU-DN	GA, AA: T2DM; AA: DFU-DN
Viswanathan *et al*[25], 2018	Indian	270 (without controls)	*SDF-1*	rs1801157	Risk factors of severe wound infections and major amputations (foot/leg)	GA + AA
Amoli *et al*[34], 2011	Iranian	586 (247 *vs* 241 *vs* 98)	*VEGF*	rs699947	Protective factor against DFU	AA
Li *et al*[35], 2018	Chinese	288 (97 *vs* 88 *vs* 103)	*VEGF*	rs699947	Protective factor against DFU	AC, AA
Li[36], 2018	Chinese	229 (121 *vs* 108) (without healthy controls)	*VEGF*	rs2010963	Protective factor against DFU	CC
Teena *et al*[42], 2020	Indian	400 (100 *vs* 150 *vs* 150)	*NRF2*	rs35652124	Risk factors of DFU	TT
Teena *et al*[43], 2021	Indian	400 (100 *vs* 150 *vs* 150)	*NRF2*	rs182428269	Protective factor against T2DM and DFU	CC, CT
					Risk factor of T2DM and DFU	TT
Peng *et al*[45], 2018	Chinese	438 (142 *vs* 148 *vs* 148)	*SIRT1*	rs12778366	Protective factor against T2DM and DF	Allele C carriers
Cao *et al*[48], 2020	Chinese	430 (128 *vs* 147 *vs* 155)	*ICAM1*	rs5498	Protective factor against T2DM and DF	GG
			ICAM1	rs3093030	Protective factor against DF	CT + TT
Li[36], 2018	Chinese	229 (121 *vs* 108) (without healthy controls)	*MCP-1*	rs1024611	Risk factor of DFU	GG
Su *et al*[51], 2018	Chinese	400 (116 *vs* 135 *vs* 149)	*MCP-1*	rs1024611	Risk factor of DFU	AG, GG
Sadati *et al*[53], 2018	Iranian	257 (123 *vs* 134)	*eNOS*	eNOS Glu298Asp	Protective factor against	TT

		(without healthy controls)			DFU	
Erdogan *et al*[37], 2018	Turkish	182 (50 *vs* 57 *vs* 75)	*eNOS*	eNOS G894T	Risk factor of T2DM but not DFU	Not related to DFU onset
Zubair and Ahmad [58], 2018	Arabian	150 (50 *vs* 50 *vs* 50)	*HSP-70*	rs2227956	Risk factor of DFU	TT
					Protective factor of DFU	CC
Pichu *et al*[60], 2015	Indian	224 (79 *vs* 79 *vs* 66)	*HIF-1α*	rs11549465	Risk factor of DFU but not T2DM	CT
Pichu *et al*[61], 2018	Indian	529 (199 *vs* 185 *vs* 145)	*HIF-1α*	rs11549467	Risk factors of T2DM and DFU	GA
Pichu *et al*[65], 2017	Indian	906 (301 *vs* 305 *vs* 300)	*LOX*	rs1800449	Risk factor of DFU but not T2DM	AA
Mrozikiewicz-Rakowska *et al*[66], 2017	Polish	670 (204 *vs* 299 *vs* 167)	*ITLN1*	rs2274907	Risk factor of DF but not T2DM	TT
Meng *et al*[68], 2017	Scottish	3394 (699 *vs* 2695)	*MAPK14*	rs80028505	Risk factor of DFU	Not reported
Wifi *et al*[71], 2017	Egyptian	90 (30 *vs* 30 *vs* 30)	*TLRs*	rs5743836	Risk factor of DFU among T2DM patients	CT
Singh *et al*[70], 2013	Indian	255 (125 *vs* 130) (DFU *vs* healthy controls)	*TLRs*	rs4986790	Risk factor of DFU	AG/GG + AG
			TLRs	rs4986791	Risk factor of DFU	TT/CT/CT + TT
			TLRs	rs11536858	Risk factor of DFU	GG/AG/GG + AG
			TLRs	rs1927914	Risk factor of DFU	CC
			TLRs	rs1927911	Risk factor of DFU	CT/CT + TT
Nehring *et al*[72], 2013	Polish	877 (122 *vs* 293 *vs* 462)	*OPG*	rs2073617	Protective factor against DF among female patients	AG
			OPG	rs2073618	Risk factor of DF among T2DM patients	CC
Soroush *et al*[76], 2017	Iranian	212 (105 *vs* 107) (without healthy controls)	*VDR*	rs2228570	Risk factor of DFU among T2DM patients	TT + CT
Zhao *et al*[78], 2015	Chinese	300 (123 *vs* 97 *vs* 80)	*FIB*	rs6056	Risk factor of DF	CT, TT

[1]This group of 270 patients included 191 patients with DFU-DN and 79 patients with DFU-peripheral vascular disease.

DF: Diabetic foot; T2DM: Type 2 diabetes mellitus; SNVs: Single Nucleotide Variations; DFO: Diabetic foot osteomyelitis; DFU-DN: Diabetic foot ulcer with diabetic neuropathy; CRP: C-reactive protein; IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor-Alpha; SDF-1: Stromal cell Derived Factor-1; VEGF: Vascular Endothelial Growth Factor; NRF2: Nuclear Factor Erythroid-2-related Factor 2; SIRT1: Sirtuin 1; ICAM1: Intercellular Adhesion Molecule 1; MCP-1: Monocyte Chemoattractant Protein-1; eNOS: Endothelial Nitric Oxide Synthase; HSP-70: Heat Shock Protein-70; HIF-1α: Hypoxia inducible factor 1 alpha; LOX: Lysyl Oxidase; ITLN1: Intelectin 1 (Omentin); MAPK14: Mitogen-activated Protein Kinase 14; TLRs: Toll-Like receptors; OPG: Osteoprotegerin; VDR: Vitamin D receptor; FIB: Fibrinogen.

extrinsic and intrinsic factors participate in DFO pathogenesis, which may also affect patient prognosis. However, considering that this was a single-center study with a limited number of participants, future multicenter studies with larger sample sizes are necessary. Additionally, the potential effects of SNVs on plasma CRP levels still remain unclear. Previous studies have reported that several CRP SNVs such as rs1800947[18], rs1205[18,19], rs3091244[20] and rs3093059[21] might play a role in the development of diseases, partially *via* their influences on plasma CRP levels. Whether CRP SNVs influence CRP levels in patients with DFU requires further investigation.

Interleukin-6

Interleukin-6 (IL-6) is an important anti-inflammatory cytokine involved in the pathogenesis of type 2 diabetes mellitus (T2DM). Dysregulations of IL-6 and IL-6 signaling have been implicated in the etiology of autoimmune and inflammatory diseases, including T2DM[22]. One of the most frequently analyzed SNV sites is rs1800795; however, there is still a dispute regarding its role in the development of DFU (Table 1).

In 2015, Dhamodharan *et al*[23] reported a potential relationship between rs1800795 and susceptibility to DFU in an Indian population. The results revealed that the allele C of rs1800795 conferred significant protection against T2DM, but not against DFU. Similar outcomes were found in a Turkish population in

a study conducted by Erdogan *et al*[24]. It was observed that the G allele of rs1800795 is a risk factor for T2DM but not an independent risk factor for DFU. In 2018, Viswanathan *et al*[25] reported that compared with genotype GG, the mutant genotypes CC and CG of rs1800795 were linked to an elevated susceptibility to *Staphylococcus sp*., *Proteus morganii*, and *Citrobacter diversus* related infections in DFU patients. This finding suggests a potential role of such an SNV in specific microbial infections. In addition, they also observed that patients with GC and CC genotypes had significantly lower IL-6 levels than those with GG genotype. This finding implies that such an SNV participates in the occurrence of severe wound infections among DFU patients, partly *via* its influence on serological IL-6 levels. A recent meta-analysis[26] focused on the potential relationship between rs1800795 and the risk of developing microvascular complications in T2DM patients. Based on a pooled analysis of 14 eligible studies, the authors concluded that rs1800795 was unrelated to susceptibility to microvascular complications of T2DM. As in this study[26], all relevant microvascular complications (diabetic nephropathy, retinopathy, and foot disease) and multiple ethnicities were included, these parameters were synthesized as a whole entity for analysis, both of which may lead to high heterogeneity, and thus, a high risk of bias to the outcomes.

Tumor necrosis factor-alpha

As part of the humoral immunity against infections, tumor necrosis factor (TNF) is involved in inflammatory responses and plays an important role in the pathogenesis of multiple infectious diseases. As one of the most prominent members of the TNF cytokine family, TNF-α is primarily secreted by macrophages, natural killer cells, lymphocytes, and neurons. Recently, increasing evidence has revealed that TNF-α SNVs are associated with the development of various inflammatory disorders, such as chronic osteomyelitis[27], coronavirus disease 2019[28], and severe sepsis[29]. Recent studies have also found that TNF-α SNVs (primarily rs1800629 and rs361525) are linked to the development of DFU (Table 1).

In a 2015 study, in addition to the *IL-6* genetic SNV, Dhamodharan *et al*[23] and colleagues also noted that TNF-α SNVs rs1800629, but not rs361525, contributed to an increased risk of developing both T2DM and DFU-DN. In 2018, this group[25] also found that both rs1800629 and rs361525 were associated with severe microbial infections. Specifically, the genotypes GA and AA of rs1800629 displayed an elevated susceptibility to *Staphylococcus sp*.-, *Proteus morganii*-, and *Citrobacter diversus*-related infections. Genotypes GA and AA of rs361525 displayed an increased risk of developing *Proteus morganii*- and *Enterococcus sp*.- associated infections. In addition, rs1800629 and rs361525 were strongly correlated with ulcer grades. The potential influence of SNV genotypes on serological levels of inflammatory biomarkers was also examined. The authors noted that patients with GA and AA genotypes of rs1800629 had significantly lower levels of TNF-α and hsCRP than those with GG genotype[25]. Nonetheless, considering that the results were derived from two studies focusing on only one Indian population and by the same study group, future studies with different populations or ethnicities are warranted.

Stromal cell-derived factor-1

Stromal cell-derived factor-1 (SDF-1) is primarily responsible for homing and migration of endothelial progenitor cells and bone marrow-derived mesenchymal stem cells. It also plays a vital role in neovascularization[30]. Considering the pathophysiological changes in DFU, a potential role for SDF-1 is probable, and it is speculated that *SDF-1* genetic SNVs may be linked to the development of DFU (Table 1).

The outcomes of a 2015 study[23] demonstrated that the allele A of SDF-1 SNV rs1801157 conferred protection against T2DM and DFU. Specifically, compared with the normal glucose tolerance (NGT) group, frequencies of the GA and AA genotypes were significantly lower in both T2DM and DFU-DN groups. In addition, the frequency of the AA genotype was significantly lower in the DFU-DN group than that in the NGT group. Multiple logistic regression analysis revealed that both genotypes displayed significant protection against T2DM. While the AA genotype alone had a protective effect against DFU-DN. Moreover, the mean glycated hemoglobin level of the AA genotype was the lowest among the three genotypes, with the highest high density lipoprotein (HDL) cholesterol level. This finding can help explain the protective effect of rs1801157 may be achieved partly *via* its influences on glycated hemoglobin and HDL-cholesterol. In a subsequent 2018 study[25], the mutant genotypes GA and AA of such an SNV site were found to be associated with an elevated risk of developing *Staphylococcus sp*.- and *Enterococcus sp*.-related infections. Additionally, this SNV was correlated with an elevated risk of major amputation, even after adjusting for confounding factors. Whether the limb can be preserved among DFU patients depends on multiple factors aside from SNVs. Thus, caution should be taken exercised in this conclusion. However, in this study[25], the authors failed to find any positive influence of SDF-1 SNV on the serum levels of the biomarkers analyzed.

Vascular endothelial growth factor

As a mitogen in vascular endothelial cells[31], vascular endothelial growth factor (VEGF) can induce collagenases and contribute to angiogenesis by clearing the matrix. This facilitates the migration and sprouting of endothelial cells[32]. VEGF regulates transforming growth factor-β and platelet-derived

growth factor during the wound healing in patients with DFU[33]. Recent studies have reported positive relationships between *VEGF* genetic SNVs and susceptibility to DFU in different populations (Table 1).

In 2011, Amoli *et al*[34] examined the potential relationship between VEGF SNVs rs25648 and rs699947, and susceptibility to DFU in an Iranian population. The results revealed that the frequency of the AA genotype of rs699947 was significantly lower in patients with DFU than in patients with diabetes without DFU. Additionally, the frequency of allele A was lower than that in the controls. These results propose that rs699947 may be a protective factor against DFU, with allele A and AA genotypes acting as protective factors. In 2018, Li *et al*[35] analyzed the potential role of VEGF SNVs rs699947 and rs13207351 in the pathogenesis of DFU in a Chinese Han cohort. They also found that allele A of rs699947 was distinctly correlated with a decreased DFU risk, with AC and AA acting as protective genotypes. However, no statistical differences were noted between rs13207351 and susceptibility to DFU in this Chinese cohort. In the same year, the same study team[36] analyzed the potential link between VEGF SNV rs2010963 and the risk of developing DFU. Specifically, the frequencies of the CC genotype and allele C of rs2010963 were lower among patients with DFU than among those with T2DM without DFU. This observation demonstrates the protective role of this particular SNV against DFU. In addition, patients with DFU with the CC genotype had significantly higher VEGF levels than those with the GG genotype. Thus, the protective effect of rs2010936 against DFU may be exerted partly *via* its influence on serological VEGF levels. In another 2018 study, Erdogan *et al*[37] analyzed the association between VEGF SNV rs3025039 and the risk of DFU development in a Turkish population. However, no significant associations were identified with either the risk of DFU development or susceptibility to T2DM. Considering the limited sample size of this study (50 DFU patients and 57 diabetic patients without DFU), the results should be interpreted with caution. Future studies with larger sample sizes are necessary.

Nuclear factor erythroid-2-related factor 2

Among diabetic patients, prolonged hyperglycemia, and oxidative stress lead to the generation of excessive reactive oxygen species (ROS). These factors contribute to endothelial dysfunction, vascular damage, and delayed wound healing[38]. In hyperglycemia, ROS levels are higher than the intrinsic antioxidant capacity. This leads to subsequent alterations in the extracellular matrix and delayed wound healing[39]. As a transcription factor, nuclear factor erythroid-2-related factor 2 (NRF2) can maintain cellular redox homeostasis and transcribe the antioxidant response element to offer endogenous protection to cells by combating ROS. Post-translational modifications of SNVs profoundly associated with diabetes have been investigated. SNVs in the regulatory motifs of the *NRF2* gene can affect its binding capacity and, thus, inhibit the transcription[40]. Epidemiological and genetic studies have indicated that NRF2 promoter SNVs in diseases are linked to oxidative stress. This indicates that NRF2 polymorphisms are genetically predisposed to disease susceptibility[41].

In a 2020 cross-sectional study conducted in an Indian population, Teena *et al*[42] examined the potential link between the NRF2 SNV rs35652124 and susceptibility to DFU. Results based on 400 participants demonstrated that the frequency of the TT genotype among the DFU patients (52%) was significantly higher than that among T2DM patients without DFU (23%) and NGT controls (12%). These observations suggest that the TT genotype might be associated with an increased risk of DFU development in both T2DM patients and healthy controls. In addition, compared with the wild CC genotype, patients with DFU with the TT genotype expressed significantly increased TNF-α and IL-6 levels but a significantly decreased IL-10 level. Increases in TNF-α and IL-6 and a decrease in IL-10 levels have been reported to slow the chronic wound healing process, especially under insulin resistance [42]. Therefore, one underlying mechanism by which NRF2 SNV rs35652124 participate in the development of DFU is through dysregulation of key genes involved in redox homeostasis and wound healing. In 2021, the same group[43] assessed the role of rs182428269 in the development of DFU in the same population. Similarly, they found that the frequency of the TT genotype of DFU subjects was the highest among the three groups (DFU patients *vs* T2DM patients without DFU *vs* NGT controls = 42% *vs* 20% *vs* 11.4%). These findings demonstrates that rs182428269 is linked to an increased susceptibility to DFU occurrence, with the TT genotype as a risk factor. Additionally, compared with the CC and CT genotypes, the expression of NRF2 was significantly decreased among the DFU subjects with the TT genotype. Thus, one potential mechanism of SNV in the development of DFU is that they may affect the expression of NRF2. Based on the outcomes of the two NRF2 SNVs studies discussed, it is speculated that dysfunction of NRF2 by SNVs might be helpful in discerning disease development and progression in T2DM.

Sirtuin 1

Sirtuin 1 (SIRT1), also known as NAD-dependent deacetylase sirtuin-1, is downregulated in patients with T2DM and is associated with oxidative stress[44]. Previous studies have indicated that SIRT1 SNVs might alter their expressions or functions and thus contribute to the development of different disorders, such as neural or vascular lesions. Recent studies have shown that SIRT1 SNVs are also involved in DFU development (Table 1).

In a 2018 case-control study, Peng *et al*[45] explored the influence of SIRT1 SNVs (rs12778366 and rs3758391) on DF susceptibility and severity in T2DM patients. Based on the outcomes of 142 DF patients, 148 T2DM patients without DF, and 148 healthy controls, they noted that the C allele of rs12778366 was correlated with reduced DF susceptibility compared to the healthy controls and T2DM patients. This study demonstrates that the allele C of rs12778366 might act as a protective factor against DF onset. Moreover, the authors noted that the DF patients displayed significant downregulation of SIRT1 expression compared to those of the T2DM patients and the healthy controls. However, no statistical differences were identified regarding SIRT1 expression among different genotypes of rs12778366. Therefore, the detailed mechanisms of SIRT1 SNVs in the pathogenesis of DF and T2DM require further investigation.

Intercellular adhesion molecule 1

Intercellular adhesion molecule 1 (ICAM1) is an important regulator of cardiovascular disorders and peripheral neuropathy in patients with diabetes[46]. It is a cell surface glycoprotein expressed in immune and endothelial cells[47]. ICAM1 is regulated by the *ICAM1* gene located at 19p13.2; its SNVs in exon regions may influence the protein expression or function. Recent studies have indicated that *ICAM1* genetic SNVs participate in DF development (Table 1).

In a 2020 study[48] comprising 128 DF patients, 147 T2DM patients, and 155 healthy controls, Cao *et al*[48] examined the potential correlations between ICAM1 SNVs rs5498 and rs3093030, and susceptibility toward DF. The results revealed that the GG genotype of rs5498 was distinctly correlated with a decreased risk of developing both T2DM and DF, with the mutant allele G acting as a protective factor. In addition, the authors analyzed the effects of ICAM1 SNVs on DF characteristics. Notably, they observed that DF patients with the GG genotype had a significantly higher levels of serum creatinine than those with the AA genotype. However, the potential reasons remain unclear. In addition to rs5498, they also reported that individuals with the rs3093030 allele T had a reduced susceptibility to DF. Thus, rs3093030 may also act as a protective factor against the onset of DF. As this study only compared outcomes from clinical data, further studies should be performed to investigate the detailed protective mechanisms.

Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2, is a potent cytokine that activates monocytes, macrophages, and lymphocytes[49]. Abnormal expression of MCP-1 may contribute to complications related to angiogenesis and vascular functions in T2DM patients[50]. Recently, growing evidence has shown that *MCP-1* genetic SNVs may be linked to DFU occurrence (Table 1).

In the aforementioned 2018 study, apart from *VEGF* SNV rs2010963, Li[36] reported the potential role of *MCP-1* SNV rs1024611 in the development of DFU. The results revealed that, compared with T2DM patients, the frequencies of both the G allele and GG genotype were increased among DFU patients. These findings implied that such a variant might be a risk factor for DFU onset among patients with T2DM. Additionally, the expression level of MCP-1 in patients with DFU with the GG genotype was significantly higher than those with the AA genotype. In the same year, Su *et al*[51] reported the potential influence of rs1024611 on the development of DFU in another Chinese cohort. Similarly, they also found that the G allele was associated with an increased risk of DFU development. Furthermore, individuals with the AG and GG genotypes had a higher risk of developing DFU. Similar findings were also obtained in that the GG genotype of rs1024611 was correlated with enhanced MCP-1 expression. This is consistent with previous findings by Li[36] that demonstrated that *MCP-1* genetic SNV rs1024611 may exert its biological effects partially *via* its influence on peripheral MCP-1 expression level. Moreover, Su *et al*[51] also found that the GG genotype of rs1024611 was correlated with a significantly higher epidermal thickness. Additionally, a significantly lower dermal thickness among patients with DFU was noted compared to those of AA and AG genotypes. This reveals another potential mechanism of such an SNV in DFU occurrence.

Endothelial nitric oxide synthase

As a key cellular signaling molecule, nitric oxide (NO) is an effective vasodilator that leads to smooth muscle relaxation. NO triggers oxidative stress by increasing free radicals and plays an important role in the pathogenesis of microvascular complications related to diabetes[52]. NO is produced through the oxidation of l-arginine by nitric oxide synthase (NOS); endothelial nitric oxide synthase (eNOS) is one of the three NOS isoforms (NOS3). Several eNOS SNVs have been linked to the occurrence of different types of disorders, including DFU (Table 1).

In a 2018 study, Sadati *et al*[53] examined associations between eNOS SNV *Glu298Asp* and the risk of DFU development in an Iranian cohort. Outcomes derived from 123 patients with DFU and 134 patients with T2DM without DFU revealed that the frequency of allele T was significantly lower in patients with DFU than in T2DM controls, with TT displaying a lower frequency in patients with DFU. This implies that the T allele may be protective against DFU. The authors explored levels of ROS and the total antioxidant power of plasma among patients with different genotypes. However, no significant

relationships were observed between such an SNV and levels of the two indicators. In another study carried out in a Turkish population, Erdogan *et al*[37] analyzed the potential effect of the eNOS SNV *G894T* on DFU susceptibility. The results revealed that the *G894T* allele T was a risk factor for diabetes but not a risk factor for DFU. As mentioned previously, considering the limited sample size of this study, future studies with more participants should be conducted.

Heat shock protein-70

Heat shock protein (HSP)-70 protein responds to stress and wound repair. Previous experiments[54,55] have shown significantly delayed or attenuated responses of cutaneous wound-induced HSP-70 expression in diabetic animals. It also functions as a key molecule in pathways linked to inflammation. Meanwhile, excessive production of inflammatory cytokines has been implicated in the pathogenesis of DFU[56]. A recent study of 946 subjects indicated that *HSP-70* genetic SNVs were strongly associated with renal complications in patients with T2DM in a South Indian population, demonstrating its possible role in T2DM and related complications.

Regarding the potential relationships between HSP-70 SNVs and DFU, a study[57] reported that HSP-70 SNVs were associated with the severity of DFU and surgical treatment outcomes. In 2018, Zubair and Ahmad[58] analyzed the potential role of HSP-70 SNV rs2227956 in the development of DFU in an Indian population. The results showed that a relatively higher frequency of the T allele was found among patients with DFU (7.3%) than among patients with T2DM (5.5%) and healthy controls (3.9%). The frequency of the TT genotype among patients with DFU was the highest (DFU *vs* T2DM *vs* healthy controls = 76% *vs* 44% *vs* 14%); and the frequency of the CC genotype among patients with DFU was the lowest (DFU *vs* T2DM *vs* healthy controls = 10% *vs* 30% *vs* 36%) among the three groups. This implies that the TT genotype may be a risk factor, whereas the CC genotype may be protective against DFU onset. Considering that only 150 participants were included (50 participants in each group), caution should be exercised in interpreting the findings.

Hypoxia inducible factor 1 alpha

Hypoxia inducible factor 1 alpha (HIF-1α) is considered a leading cause of various chronic diseases, including diabetes. It is a key regulator of genes involved in cellular response to hypoxia[59]. Growing evidence has shown that *HIF-1α* gene SNVs may be related to the development of DFU (Table 1).

In a 2015 study, Pichu *et al*[60] analyzed the potential link between HIF-1α SNV rs11549465 and the risk of developing DFU in an Indian population. The results confirmed that the frequencies of the CT genotype in both patients with T2DM and patients with DFU were higher than those in healthy controls. However, a significant difference was only found among the patients with DFU. This suggests that the CT genotype might be a risk factor for DFU but not for T2DM. The outcomes of subsequent analyses demonstrated that HIF-1α expression in patients with DFU was lower than that in patients with T2DM and healthy controls. In addition, patients with DFU with the CT genotype had a lower expression level of HIF-1α than those with the CC genotype. This observation implied that reduced HIF-1α expression might be associated with the development of DFU. In 2018, the same study[61] examined the role of HIF-1α SNV rs11549467 in DFU occurrence. The frequencies of the GA genotype were significantly higher in patients with T2DM and DFU than in healthy controls. Thus, this genotype was considered a risk factor for both T2DM and DFU onset. Similar to their previous study[60], a decreased expression level of HIF-1α was found among the patients with DFU compared to that in patients with T2DM and healthy controls. These findings suggest that HIF-1α may play an important role in DFU pathogenesis. However, in-depth mechanistic studies are required.

Lysyl oxidase

Lysyl oxidase (LOX), an extracellular matrix-modifying enzyme, is associated with cell proliferation, metastasis, angiogenesis, and wound healing. Elevated expression of the *LOX* gene and accompanying cross-linked collagen fibrils in diabetic skin may lead to changes in tissue mechanical properties. These features are important for the regulation of tensile and elastic features of connective tissues[62,63]. LOX expression may be positively regulated by high glucose levels in diabetic skin[64]. LOX SNVs have also been associated with DFU development (Table 1).

In a 2017 case-control study, Pichu *et al*[65] analyzed the potential relationship between LOX SNV rs1800449 and susceptibility to DFU in an Indian population. The outcomes of 906 participants showed a significantly higher frequency of allele A among the DFU patients (42 %) than that among the controls (33%), with the AA genotype as a risk factor for DFU. Moreover, the LOX transcript level linked to the AA genotype among patients with DFU was significantly higher than that of the AA genotype among patients with T2DM and controls. This suggests that the increased expression of LOX may participate in the onset of DFU.

Intelectin 1

Intelectin 1 (ITLN1), also known as omentin, is encoded by the *ITLN1* gene located on the long arm of chromosome 1 (1q21.3)[66]. Mrozikiewicz-Rakowska *et al*[66] examined the potential role of rs2274907 in the development of DFU in a Polish population. Based on 670 individuals, they found that the T allele

was more frequent in the DF group than in the control group. Therefore, the TT genotype is a possible risk factor. In addition, this effect was sex-specific and observed in males (Table 1). Although the influence of such an SNV on the concentration of omentin in the DFU patients remains unclear, the authors introduced the underlying mechanisms regarding the protective effects of omentin on endothelium and smooth muscle cells for detail[66]. Omentin is able to stimulate NO production, leading to the endothelium–dependent vasodilation. In addition, omentin can also suppress the inflammatory response in endothelial cells by inhibiting the c-Jun N-terminal kinase activation *via* the AMP-activated protein kinase/eNOS signaling pathway. Furthermore, omentin decreases the adhesion of monocytes to endothelial cells by reducing expression of vascular cell adhesion protein-1 on the surface of monocytes as well as reducing the expression of intercellular adhesion molecule-1. Aside from endothelium, omentin also displayed an inhibitory effect on TNF–α–induced adhesion of monocytes in vascular smooth muscle cells of the rat. Nonetheless, the detailed mechanisms of ITLN1 SNVs in the development of DFU are still largely unknown and requires further research.

Mitogen-activated protein kinase 14

Mitogen-activated protein kinase 14 (MAPK14) targets a broad range of nuclear and cytosolic substrates that participate in a wide variety of cellular processes, such as proliferation, differentiation, apoptosis, transcription regulation, and development. It is a kinase involved in cellular responses to extracellular stimuli, such as pro-inflammatory cytokines or physical stress[67]. In a 2017 study, Meng *et al*[68] analyzed potential SNVs related to the development of DFU in a Scottish population. The results showed that rs80028505 was associated with increased susceptibility to DFU in a Scottish cohort (Table 1).

Toll-like receptors

Toll-like receptors (TLRs) superfamily members play a fundamental role in detecting invading pathogens or damage and initiating the innate immune system. Aberrant activation of TLRs exaggerates T cell-mediated autoimmune activation, causing unwanted inflammation and promoting DFU[69]. Recent studies have indicated that TLR SNVs are involved in DFU development (Table 1).

In a 2013 study, Singh *et al*[70] reported potential associations between TLR4 SNVs (rs4986790, rs4986791, rs11536858, rs1927911, and rs1927914) and susceptibility to DFU in an Indian population study. The results showed that these TLR4 SNVs correlated with an increased risk of developing DFU. They also reported 15 haplotypes with a frequency greater than 1%, and outcomes revealed that the haplotype ACATC displayed a strong association with DFU risk. In contrast, the haplotypes ATATC and ATGTT were noted to be protective against DFU. Furthermore, the authors also introduced two different models to predict the risk of DFU development. They proposed that the artificial neural network model was better than the multivariate linear regression model. In 2017, Wifi *et al*[71] analyzed the relationship between *TLR2* (rs3804100) and *TLR9* (rs5743836) SNVs and the risk of developing DF in an Egyptian population. The results suggest that rs5743836, rather than rs3804100, is associated with an elevated risk of DFU development among patients with T2DM. However, considering the limited number of eligible participants, cautious attitudes should be taken towards inferring the outcomes and conclusions.

Osteoprotegerin

Osteoprotegerin (OPG) plays a key role in the regulation of bone resorption and it belongs to the TNF superfamily. In a 2013 study, Nehring *et al*[72] examined the links between three SNVs (rs2073617, rs2073618, and rs3134069) located in the *TNFRSF11B* gene and the risk of DF development in a Polish population. The results showed that the C allele and CC genotype of rs2073618 were risk factors for DF onset in T2DM patients. For rs2073617, the mutant allele A and AG genotypes were protective against DF (Table 1).

Vitamin D receptor

Growing evidence has demonstrated that vitamin D receptor (VDR) SNVs are involved in the pathogenesis of several inflammatory disorders, such as fracture-related infection[73], tuberculosis[74], and periodontitis[75]. In a 2017 study, Soroush *et al*[76] analyzed the role of VDR SNV rs2228570 in the development of DFU in an Iranian population. The results showed that the frequencies of genotypes TT and TC among patients with DFU were significantly higher than those without DFU. This finding implies that such genotypes of this SNV present a risk factor to this cohort. In addition, they also evaluated the expression levels of oxidative stress indicators, thiobarbituric acid reactive substances (TBARS), and ferric-reducing ability of plasma (FRAP) among different genotypes of the SNV. The results showed that the median level of TBARS among patients with the TT and TC genotypes was significantly higher than that of the CC genotype. However, no statistical difference in FRAP levels between the two groups was noted. Nonetheless, no significant relationships were found between the genotypes and TBARS or FRAP levels among healthy controls. This suggests that one underlying mechanism of VDR SNV rs2228570 in DFU pathogenesis is partly *via* its influence on TBARS levels (Table 1).

Fibrinogen

Fibrinogen (FIB) and fibrin play important roles in multiple biological processes, including fibrinolysis, blood clotting, inflammation, wound healing, cellular and matrix interactions, and neoplasia. A recent study[77] confirmed the definitive role of FIB as a promising inflammatory marker in the discrimination of DFU. In a 2015 study, Zhao *et al*[78] investigated the correlation between *FIB* SNV rs6056 polymorphism and susceptibility towards DF in a Chinese population. Outcomes based on 300 subjects demonstrated that the mutant allele T, CT, and TT genotypes were risk factors for DF onset, following univariate logistic regression analysis. The TT genotype was associated with a relatively higher serological FIB level (Table 1).

LIMITATIONS AND FUTURE PERSPECTIVES

Increasing evidence has suggested that, in addition to extrinsic factors, intrinsic factors such as SNVs also participate in the development of DFU. However, these investigations had limitations. First, the sample sizes of most studies were limited; therefore, caution should be exercised regarding inferring relevant outcomes and conclusions. Second, most of the studies were conducted in Asian countries (*e.g.*, India, China, and Iran). To comprehensively evaluate the potential roles of SNVs in the pathogenesis of DFU, investigations focusing on different populations or ethnicities should be conducted in the future. Third, as the majority of the analyzed studies only reported preliminary findings based on case-control comparison outcomes, there is still a lack of in-depth research on mechanisms.

Based on these limitations, future studies should focus on two primary aspects. On the one hand, multi-center studies with larger sample sizes and diverse populations should be conducted. This will ensure a more accurate and comprehensive assessment of the potential roles of SNVs in the development of DFU. On the other hand, the detailed mechanisms should be investigated from different perspectives for SNVs with clinical significance.

CONCLUSION

Based on recent findings, SNVs located in the genes of *CRP* (rs11265260, rs1800947, rs2794520, rs1130864, rs3093059), *IL-6* (rs1800795), *TNF-α* (rs1800629, rs361525), *SDF-1* (rs1801157), *VEGF* (rs699947, rs2010963), *NRF2* (rs35652124, rs182428269), *SITR1* (rs12778366), *ICAM1* (rs5498, rs3093030), *MCP-1* (rs1024611), *eNOS* (Glu298Asp), *HSP-70* (rs2227956), *HIF-1α* (rs11549465, rs11549467), *LOX* (rs1800449), *ITLN1* (rs2274907), *MAPK14* (rs80028505), *TLRs* (rs5743836, rs4986790, rs4986791, rs11536858, rs1927914), *OPG* (rs2073617, rs2073618), *VDR* (rs2228570), and *FIB* (rs6056) may be important molecular players influencing the development and progression of DFU.

FOOTNOTES

Author contributions: Hu YJ and Song CS contributed equally to this study; Hu YJ and Jiang N conceived and designed the study; Song CS searched the literature; Song CS and Jiang N drafted the article; Hu YJ, Song CS, and Jiang N revised the manuscript; All authors approved the final version of the submitted article.

Supported by National Natural Science Foundation of China, No. 82172197; Guangdong Basic and Applied Basic Research Foundation, No. 2022A1515012385; and Guangdong Provincial Science and Technology Project, No. 2020A0505100039.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1154-1167

DOI: 10.4239/wjd.v13.i12.1154

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Observational Study

Baseline moderate-range albuminuria is associated with protection against severe COVID-19 pneumonia

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Specialty type: Medicine, general and internal

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Diaconu CT, Romania; Elfaki I, Saudi Arabia

Received: August 10, 2022
Peer-review started: August 10, 2022
First decision: October 5, 2022
Revised: October 18, 2022
Accepted: December 1, 2022
Article in press: December 1, 2022
Published online: December 15, 2022

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Abstract

BACKGROUND

Diabetes mellitus is considered a leading contributor to severe coronavirus disease 2019 (COVID-19).

AIM

To characterize differences between hospitalized diabetic patients with *vs* without COVID-19, and parameters associated with COVID-19 severity for prediction.

METHODS

This case-control study included 209 patients with type 2 diabetic mellitus hospitalized at the Galilee Medical Center (Nahariya, Israel) and recruited between September 2020 and May 2021, 65 patients with COVID-19 infection in dedicated wards and 144 COVID-19-negative patients in internal medicine wards hospitalized due to other reasons. Clinical parameters - including age, type of antiglycemic medications, presence of retinopathy, smoking history, body mass index (BMI), glycosylated hemoglobin, maximum neutrophil:lymphocyte ratio

(NLR_{max}), C-reactive protein (CRP), estimated glomerular filtration rate (eGFR), and albumin (blood and urine) - were compared between the two primary patient groups, and then between COVID-19-negative patients hospitalized due to infectious *vs* non-infectious disease. Finally, we explored which parameters were associated with severe COVID-19 pneumonia.

RESULTS

COVID-19-negative patients were older (63.9 ± 9.9 *vs* 59.8 ± 9.2, $P = 0.005$), and had longer duration of diabetes ($P = 0.031$), lower eGFR ($P = 0.033$), higher albumin ($P = 0.026$), lower CRP ($P < 0.001$), greater smoking prevalence ($P < 0.001$), and more baseline albuminuria (54.9% *vs* 30.8%, $P = 0.005$) at admission; 70% of COVID-19 patients with albuminuria had moderate-range albuminuria (albumin:creatinine 30-300 mg/g). Most of the patients with albuminuria had chronic kidney disease stage II (CKD II). Oral antiglycemic therapies were not significantly different between the two groups. Multivariable logistic regression showed that higher BMI was significantly associated with severe COVID-19 (OR 1.24, 95%CI: 1.01-1.53, $P = 0.04$), as was higher NLR_{max} (OR 1.2, 95%CI: 1.06-1.37, $P = 0.005$). Surprisingly, pre-hospitalization albuminuria, mostly moderate-range, was associated with reduced risk (OR 0.09, 95%CI: 0.01-0.62, $P = 0.015$). Moderate-range albuminuria was not associated with bacterial infections.

CONCLUSION

Moderate-range albuminuria in COVID-19-positive diabetic patients with CKD II is associated with less severe COVID-19. Further studies should explore this potential biomarker for risk of COVID-19-related deterioration and early interventions.

Key Words: Diabetes mellitus; COVID-19; Albuminuria; Severity; Chronic kidney disease; Immunomodulation

Core Tip: Type 2 diabetes mellitus and its risk factors are considered to be contributors to severe coronavirus disease 2019 (COVID-19). In this study, we analyzed our single-center clinical data of adults with type 2 diabetes between September 2020 and May 2021 to determine the impact of risk factors on severity of COVID-19 pneumonia. Surprisingly, we found that moderate-range pre-hospitalization albuminuria was associated with reduced risk of severe COVID-19 pneumonia. Further studies are needed to explore this association and pathogenesis relating to immunomodulation, which may indicate a biomarker for patients at reduced risk for COVID-19-related deterioration that may translate to therapeutic interventions.

Citation: Bashkin A, Shehadeh M, Shbita L, Namoura K, Haiek R, Kuyantseva E, Boulos Y, Yakir O, Kruzel-Davila E. Baseline moderate-range albuminuria is associated with protection against severe COVID-19 pneumonia. *World J Diabetes* 2022; 13(12): 1154-1167
URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1154.htm
DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1154

INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the causative pathogen for coronavirus disease 2019 (COVID-19) pneumonia[1]. COVID-19 infection can cause various symptoms of varying severity, starting from mild disease with upper respiratory tract infection and continuing to moderate and severe pneumonia with a systemic inflammatory response syndrome, acute respiratory distress syndrome (ARDS), multi-organ involvement, and shock[2].

Several risk factors for severe COVID-19 disease have been described, and include advanced age, male sex, smoking history, and underlying chronic diseases such as cardiovascular disease (CVD), diabetes mellitus, obesity, underweight, and chronic kidney disease (CKD) [defined as estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m²], as well as socioeconomic deprivation[3,4]. The presence of diabetes and the individual degree of hyperglycemia appear to be independently associated with COVID-19 severity and increased mortality[5].

Given the compromised immune function in patients with diabetes, especially innate immunity with impaired natural killer (NK) cells[5-7], impaired T cell responses with increased Th1 and Th17 cells, reduced T regulatory cells, and altered cytokine response[8], diabetes is considered to be a risk factor for

severe COVID-19 pneumonia[5,9]. A recent retrospective study demonstrated elevated cytokines, imbalance of Th1/Th2 secreted cytokines and reduced levels of CD8+ T cells and NK cells in patients with diabetes suffering from COVID-19 pneumonia compared to patients without diabetes, with reduced level of CD8+ T cells and NK cells being more pronounced in non-survivors[10]. In addition, other risk factors for severe COVID-19 pneumonia are associated with diabetes, *e.g.*, obesity, hyperglycemia, CVD, and CKD[1-3].

In this case-control study, we aimed to characterize the differences between patients with diabetes hospitalized in internal medicine departments and patients with diabetes suffering from COVID-19 pneumonia in designated wards at the Galilee Medical Center. Among the patients with COVID-19 infection, we explored clinical parameters that were associated with severe COVID-19 pneumonia for predictive value.

MATERIALS AND METHODS

Participants

Data were analyzed from 209 type 2 diabetic patients hospitalized at the Galilee Medical Center between September 2020 and May 2021 and participating in a study evaluating the prevalence of and related factors for *de novo* positive COVID19 serology (ethical committee approval number 0073-21-NHR, dated 05-Jul-2021). Sixty-five patients suffering from COVID-19 infection were hospitalized in the COVID-19 wards and 144 patients with other diseases were hospitalized in the internal medicine wards, the latter recruited concurrently for unbiased comparison (Figure 1). Diabetes was defined by glycosylated hemoglobin (HbA1c) ≥ 6.5% and by medical history of type 2 diabetes diagnosis in the past. Patients hospitalized in the internal medicine wards were recruited during their hospitalization period, while patients suffering from COVID-19 pneumonia were recruited after their discharge from the hospital. All participants signed the informed consent form as approved by the Galilee Medical Center Helsinki Committee (investigational review board). The total number of participants was reached per enrollment criteria of patients hospitalized during the selected time period.

Design and procedures

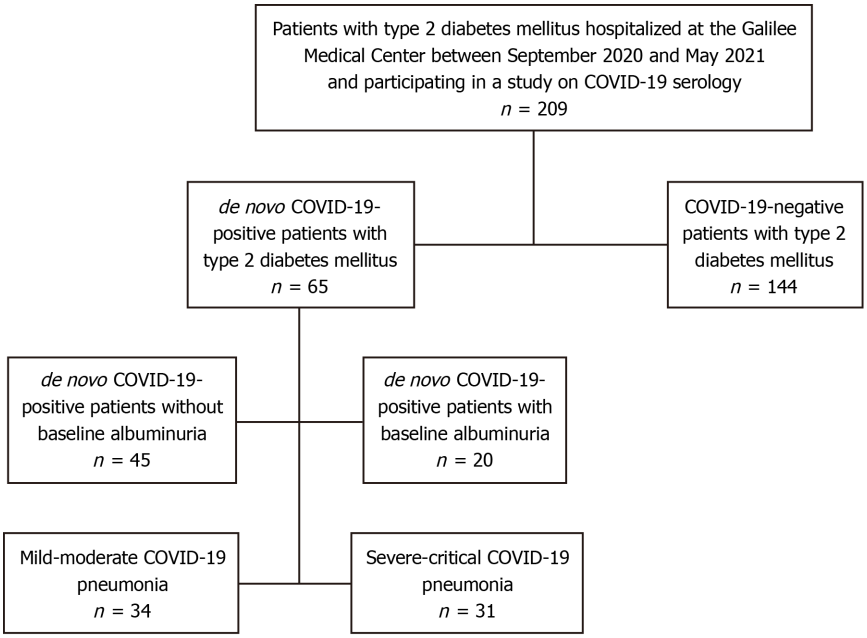
This case-control study included the following two parts: (1) First we compared the clinical parameters between the two primary patient groups, followed by a comparison of the clinical characteristics between patients hospitalized due to infectious *vs* non-infectious disease in the internal medicine wards; and (2) Second, we explored which clinical parameters were associated with severe COVID-19 pneumonia.

Demographic, clinical, and laboratory parameters were collected from electronic hospital and community records using Chameleon and Ofek software, respectively. The following baseline parameters were recorded: Age, sex, religion, type of antiglycemic medications [metformin, dipeptidylpeptidase-4 (DPP-4) inhibitors, sulfonylurea, sodium glucose cotransporter-2 (SGLT2) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, and insulin], presence of retinopathy, smoking history, body mass index (BMI), and HbA1c. The following parameters were collected during the hospitalization period: maximum neutrophil:lymphocyte ratio (NLR_{max}) and C-reactive protein (CRP) (plus values at admission), eGFR, and albumin. COVID-19 infection was defined as a positive SARS-CoV-2 polymerase chain reaction.

Baseline moderate-range albuminuria was defined as an albumin-to-creatinine ratio between > 30 and < 300 mg/g in two urine analyses performed during the 18 mo prior to hospitalization; macroalbuminuria was defined as > 300 mg/g. eGFR was calculated by using the CKD Epidemiology Collaboration creatinine equation[11]. Baseline HbA1c and eGFR were calculated as the average of up to two last values of the respective tests during the 12 mo prior to hospitalization. Retinopathy was defined according to fundoscopic examination conducted during the 18 mo prior to hospitalization (it included background retinopathy, proliferative retinopathy, or macular edema).

Quantitative variables other than albuminuria were not divided into subgroups. Albuminuria was divided dichotomously for patients with and without, based on known categorization by urine-albumin-to-creatinine ratio (< 30 mg/g and ≥ 30 mg/g); and for those with, further divided into three groups according to albuminuria severity (albuminuria < 30 mg/g, 30-300 mg/g, and > 300 mg/g).

The severity of COVID-19 infection was determined in accordance with the following Israel Ministry of Health criteria published July 12, 2020 (according to the United States National Institutes of Health): (1) Mild illness when there are symptoms of mild viral upper respiratory tract infection; (2) Moderate illness per imaging and oxygen saturation ≥ 94% on room air; (3) Severe illness when one of the following criteria were met: respiratory rate > 30 breaths/minute, oxygen saturation < 94% on room air, PaO_2/FiO_2 < 300 mmHg, or lung infiltration > 50%; and (4) Critical illness per hemodynamic instability, need for mechanical ventilation, and/or multiorgan failure.



DOI: 10.4239/wjd.v13.i12.1154

Figure 1 Enrolled participants and analysis groups. COVID-19: Coronavirus disease 2019.

Statistical analysis

Quantitative variables were analyzed for mean (SD), median, and interquartile range (IQR). Categorical variables were analyzed with frequencies and percentages.

Differences between groups for continuous variables were compared using independent sample *t*-test or Mann-Whitney test. We chose independent sample *t*-test when the compared variables did not deviate significantly from the normal distribution. Differences between groups for categorical variables were compared with Chi-square or Fisher's exact tests (if expectancy < 5).

Correlations between continuous variables were examined with Spearman's correlation coefficient test, which was chosen over Pearson's correlation coefficient test according to the variable's distribution shape. Multivariable logistic regression modelling was used to determine the risk factors for severe or critical COVID-19 and separately for infectious compared to non-infectious disease in patients without COVID-19 infection. In the multivariable analysis, the severity of COVID-19 pneumonia and presence of infectious disease were the dependent variables, while the following baseline parameters were independent variables: age, sex, BMI, last eGFR measured before hospitalization, HbA1c, NLR_{max}, and albuminuria before hospitalization. These risk factors were chosen according to the univariable analysis results and theoretical considerations. We defined the following three models according to variables: Model 1 included sex, age, and BMI; Model 2 included Model 1 variables plus HbA1c, eGFR, and NLR_{max}; and Model 3 included Model 2 variables plus the presence of albuminuria. Model 3 presents an adjustment of background and clinical measures (*e.g.*, age, eGFR, NLR_{max}, *etc.*) for the albuminuria variable.

Odds ratios (OR) and 95% confidence intervals (CI) for OR are provided as estimates of risk for each variable.

Analyses were performed with IBM SPSS Statistics software version 27.0 (Chicago, IL, United States). A P value of < 0.05 was considered statistically significant. Two-sided P values are presented unless otherwise specified.

RESULTS

Between September 2020 and May 2021, 65 patients with diabetes suffering from COVID-19 infection and 144 diabetic COVID-19-negative patients hospitalized due to other reasons were enrolled in this study.

Clinical characteristics of patients with diabetes, with and without COVID-19 infection

Clinical characteristics of patients with diabetes, with COVID-19 infection compared to diabetic patients without COVID-19 infection, are presented in Table 1. Patients without COVID-19 were older than patients with COVID-19 (63.9 ± 9.9 years compared to 59.8 ± 9.2 years, respectively, P = 0.005), had lower prevalence of smoking (6.2% compared to 33.3%, P < 0.001), longer duration of diabetes (P =

Table 1 Clinical and demographic characteristics of patients with type 2 diabetes, with and without coronavirus disease 2019 infection

	Patients without COVID-19 infection, *n* = 144	Patients with COVID-19 infection, *n* = 65	*P* value
Age (yr), mean (SD)	63.9 (9.9)	59.8 (9.2)	0.005[1]
Median (IQR)	65 (57-71)	61 (53-66)	
Sex, female, *n* (%)	50 (34.7)	32 (49.2)	0.066[2]
Population group, *n* (%)			0.067[2]
Jews	63 (44.7)	20 (30.8)	
Arabs	78 (55.3)	45 (69.2)	
BMI (kg/m^2), mean (SD)	30.7 (5.9)	32.1 (4.6)	0.09[1]
Median (IQR)	30.3 (27.4-33.8)	31.6 (29.1-34.7)	
Diabetes duration (yr), median (IQR)	13 (8-17.8)	10 (5.5-14.5)	0.031[3]
eGFR (mL/min/1.73 m^2 body surface area) at baseline, median (IQR)	85.4 (62.2-97.6)	91.9 (75.3-101.0)	0.033[3]
HbA1c (%), median (IQR)	7.6 (6.5-9.1)	7.4 (6.6-9.1)	1.00[3]
Metformin, *n* (%)	110 (76.9)	56 (86.2)	0.14[2]
DPP-4 inhibitors, *n* (%)	29 (20.1)	18 (27.7)	0.28[2]
Sulfonylurea, *n* (%)	8 (5.6)	7 (10.8)	0.25[4]
SGLT2 inhibitors, *n* (%)	36 (25.0)	18 (27.7)	0.73[2]
GLP-1 agonists, *n* (%)	25 (17.4)	12 (18.5)	1.00[2]
Basal insulin, *n* (%)	60 (41.7)	18 (27.7)	0.064[2]
Prandial insulin, *n* (%)	30 (21.0)	6 (9.2)	0.047[2]
Current smoking, *n* (%)	48 (33.3)	4 (6.2)	< 0.001[2]
No albuminuria, *n* (%)	65 (45.1)	45 (69.2)	0.002[2]
Albuminuria < 30 mg/g, *n* (%)	79 (54.9)	20 (30.8)	0.005[3]
Albuminuria 30-300 mg/g, *n* (%)	65 (45.1)	14 (21.5)	
Albuminuria > 300 mg/g, *n* (%)	14 (9.7)	6 (9.2)	
Retinopathy, *n* (%)	21 (21.4)	7 (15.2)	0.50[2]
NLR_{max} at hospitalization, median (IQR)	4.0 (2.5-7.8)	6.5 (2.6-10.0)	0.12[3]
CRP (mg/L) at admission, median (IQR)	9.7 (4.8-45.4)	71.8 (12.2-145.9)	< 0.001[3]
CRP_{max} (mg/L) at hospitalization, median (IQR)	11.3 (5.2-68.1)	86.6 (12.2-167.6)	< 0.001[3]
eGFR (mL/min/1.73 m^2 body surface area) at hospitalization, median (IQR)	86.0(62.2-96.0)	92.1(76.2-100.8)	0.030[3]
Albumin, median (IQR)	3.8 (3.4-4.0)	3.6(3.2-3.9)	0.026[1]

[1]Independent sample *t*-test.
[2]Chi-square test.
[3]Mann-Whitney test.
[4]Fisher's exact test.
COVID-19: Coronavirus disease 2019; IQR: Interquartile range; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; eGFR: Estimated glomerular filtration rate; DPP-4: Dipeptidyl-peptidase 4; SGLT2: Sodium/glucose cotransporter 2; GLP-1: Glucagon-like peptide 1; NLR_{max}: Maximum neutrophil:lymphocyte ratio; CRP: C-reactive protein; CRP_{max}: Maximum C-reactive protein.

0.031), lower eGFR (P = 0.033), higher albumin (P = 0.026), and lower CRP at admission, as well as lower maximum value of CRP (CRP_{max}) during hospitalization (P < 0.001). Interestingly, baseline albuminuria was more common in patients without COVID-19 infection (54.9% compared to 30.8%, P = 0.005). There was a trend toward a higher percentage of insulin therapy in patients without COVID-19 (P = 0.047 and P = 0.064 for prandial and basal insulin, respectively). Use of other antiglycemic therapies were not significantly different between the two groups.

Clinical characteristics of patients with diabetes suffering from COVID-19, with and without albuminuria

Among the 65 patients suffering from COVID-19 infection in this cohort, 20 had documented albuminuria prior to hospitalization, whereas 45 did not. Most of the patients with albuminuria had CKD II and moderately increased albuminuria (A2) (Table 2). Basal insulin therapy was more common in patients with albuminuria (50% compared to 17.8%, P = 0.015). As expected, patients with albuminuria had significantly higher values of HbA1c (median 8.9% and IQR_{25-75} 7.3%-10.4%, compared to 7.2% and 6.6%-8.4%, respectively, P = 0.02). Other parameters were not significantly different between the two groups (Table 2).

Predictors of severe COVID-19 pneumonia

Univariable analysis demonstrated increased risk for severe COVID-19 pneumonia with higher inflammatory markers NLR_{max} and CRP (P < 0.001) and lower albumin level (P < 0.001). Oral antiglycemic therapies were not significantly different between patients with moderate or severe pneumonia (Table 3).

The following variables were considered to be confounders due to putative correlation with albuminuria and COVID-19 severity: age, BMI, eGFR, HbA1c, and NLRmax. We therefore examined the correlation between each of these variables and albuminuria (see Table 2) and COVID-19 severity (see Table 3). According to the univariable analysis (Tables 2 and 3), HbA1c was found to be significant correlated with albuminuria (P = 0.02), but was not found to be significant in the COVID-19 severity univariable analysis (P = 0.93); NLR_{max} was not found to be correlated with albuminuria (P = 0.48), but was found to be correlated with COVID-19 severity (P < 0.001); age and BMI were found only to trend toward correlations with albuminuria (P = 0.16 and P = 0.19 respectively) and COVID-19 severity (P = 0.26, P = 0.22), possibly due to the small sample size; eGFR was not found to correlate with either albuminuria or COVID-19 severity (P = 0.90 and P = 0.78, respectively).

Because of the theoretical consideration and the above findings, we decided to include those variables in the multivariable analysis. Given the association between sex and COVID-19 severity reported in the literature, we included this variable in the univariable analysis, wherein male sex showed only a trend toward correlation with COVID-19 severity (P = 0.14), with no correlation for albuminuria (P = 0.60).

Variables associated with severe COVID-19 pneumonia in the multivariable logistic analysis according to the Models 1-3 are presented in Table 4, and the multivariable regression in Model 3 is depicted in Figure 2. The dependent variable is the severity of COVID-19 pneumonia, while the following parameters are independent variables: age, sex, BMI, last eGFR measured before hospitalization, HbA1c, NLR_{max}, and albuminuria before hospitalization. In the final model, as expected, a higher BMI was significantly associated with severe COVID-19 pneumonia (OR 1.24, 95%CI: 1.01-1.53, P = 0.04), as was higher NLR_{max} (OR 1.20, 95%CI: 1.06-1.37, P = 0.005). Surprisingly, the presence of moderate-range albuminuria before hospitalization was associated with reduced risk (OR 0.09, 95%CI: 0.01-0.62, P = 0.015). Of note, 70% of COVID-19 patients with proteinuria had moderate-range albuminuria.

As expected, moderate correlation strength was found between age and diabetes duration in the COVID-19 group (Spearman's correlation coefficient test r = 0.58, P < 0.001). Given this correlation, only age was included in the multivariable regression model. Similarly, moderate-to-strong correlation strength was found between NLR_{max} and CRP_{max} in the COVID-19-positive patients (Spearman's correlation coefficient test r = 0.59, P < 0.001 for the COVID-negative patients, and r = 0.73, P < 0.001 for the COVID-19-positive patients). Given the significant correlation between CRP and NLR_{max}, only NLR_{max} was included in the multivariable regression model.

Moderate range albuminuria was not associated with bacterial infections

Given the surprising protective association between moderate-range albuminuria and severe COVID-19 infection, we wanted to explore whether this association is similarly observed in bacterial infections. We hypothesized that this protective association is specific for viral infections such as COVID-19 and not to bacterial infections. For this, similar multivariable logistic regression models were conducted in COVID-19-negative patients without bacterial infections *vs* patients with bacterial infections to characterize which variables are associated with the latter. Variables associated with the absence *vs* presence of bacterial infection in the multivariable analysis according to the Models 1-3 are presented (Table 5). In the final model, NLR_{max} was significantly associated with bacterial infection. The protective effect of albuminuria was not observed with regard to bacterial infection. The apparent protective effect of moderate-range albuminuria in patients with CKD II was specific to COVID-19 infection in this cohort, but may be relevant to other viral infections as well.

DISCUSSION

Diabetes mellitus has been associated with severe COVID-19 pneumonia[3-5,8]. Hyperglycemia increases SARS-CoV-2 replication in human monocytes, and glycolysis sustains SARS-CoV-2 replication

Table 2 Clinical and demographic characteristics of patients type 2 diabetes and coronavirus disease 2019 infection, with and without albuminuria

	Diabetic patients without baseline albuminuria, *n* = 45	Diabetic patients with baseline albuminuria, *n* = 20	*P* value
Age (yr), mean (SD)	58.7 (9.2)	62.2 (9.0)	0.16[1]
Median (IQR)	60.0 (50.5-65.0)	63.0 (56.5-68.8)	
Sex, female, *n* (%)	21 (46.7)	11 (55.0)	0.60[2]
Population group, *n* (%)			0.77[2]
Jews	13 (28.9)	7 (35.0)	
Arabs	32 (71.1)	13 (65.0)	
BMI (kg/m^2), mean (SD)	31.6 (4.5)	33.2 (4.6)	0.19[1]
Median (IQR)	30.9 (28.6-33.7)	32.3 (29.7-35.9)	
Diabetes duration (yr), median (IQR)	10.0 (5.5-14.0)	11.0 (5.3-17.0)	0.25[1]
eGFR (mL/min/1.73 m^2 body surface area) at baseline, median (IQR)	90.5 (77.8-99.5)	95.3 (69.6-101.1)	0.90[3]
HbA1c (%), median (IQR)	7.2 (6.6-8.4)	8.9 (7.3-10.4)	0.02[3]
Metformin, *n* (%)	41 (91.1)	15 (75)	0.12[4]
DPP-4 inhibitors, *n* (%)	15 (33.3)	3 (15)	0.15[2]
Sulfonylurea, *n* (%)	5 (11.1)	2 (10)	1.00[4]
SGLT2 inhibitors, *n* (%)	15 (33.3)	3 (15)	0.15[2]
GLP-1 agonists, *n* (%)	8 (17.8)	4 (20)	1.00[4]
Basal insulin, *n* (%)	8 (17.8)	10 (50)	0.015[2]
Prandial insulin, *n* (%)	2 (4.4)	4 (20)	0.067[4]
Current smoking, *n* (%)	2 (4.4)	2 (10)	0.58[4]
Retinopathy, *n* (%)	4 (12.9)	3 (20)	0.67[4]
NLR_{max}, median (IQR)	6.2 (2.5-9.4)	6.7 (2.6-18.0)	0.48[3]
CRP (mg/L) at admission, median (IQR)	90.4 (10.9-153.6)	43.9 (12.5-106.8)	0.33[3]
CRP_{max} (mg/L) at hospitalization, median (IQR)	92.8 (11.4-171.3)	61.0 (12.5-157.3)	0.60[3]
eGFR (mL/min/1.73 m^2 body surface area) at hospitalization, median (IQR)	90.3 (77.0-98.4)	94.3 (67.8-101)	0.76[3]
Albumin, median (IQR)	3.6 (3.3-4.0)	3.6 (3.1-3.9)	0.25[1]

[1]Independent sample *t*-test.
[2]Chi-square test.
[3]Mann-Whitney test.
[4]Fisher's exact test.
IQR: Interquartile range; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; eGFR: Estimated glomerular filtration rate; DPP-4: Dipeptidyl-peptidase 4; SGLT2: Sodium/glucose cotransporter 2; GLP-1: Glucagon-like peptide 1; NLR_{max}: Maximum neutrophil:lymphocyte ratio; CRP: C-reactive protein; CRP_{max}: Maximum C-reactive protein.

via the production of mitochondrial reactive oxygen species and activation of hypoxia-inducible factor 1 α[12]. Further, individuals suffering from diabetes are thought to have chronic low-grade inflammation, which might facilitate the cytokine storm that can lead to clinical deterioration of COVID-19 patients [13]. In addition, patients with diabetes have impaired NK cell activity and altered T cell subpopulations that may increase the susceptibility to severe COVID-19 pneumonia[5,6,9,13,14]. Moreover, other risk factors for severe COVID-19 pneumonia are associated with diabetes, *e.g.*, obesity, CVD, and CKD[5,8, 10]. Therefore, in the current study, we focused on patients with type 2 diabetes who were hospitalized at the Galilee Medical Center due to COVID-19 infection or other acute diseases for comparison.

Interestingly, baseline albuminuria was more common in patients without COVID-19 infection (54.9% compared to 30.8%, $P = 0.005$). The observed low rate of albuminuria in the COVID-19 group led us to explore the associations of several baseline clinical variables, including baseline albuminuria and

Table 3 Clinical and demographic characteristics of patients with type 2 diabetes, with mild or moderate versus severe or critical coronavirus disease 2019 pneumonia

	Mild-moderate COVID-19 pneumonia, *n* = 34	Severe-critical COVID-19 pneumonia, *n* = 31	*P* value
Age (yr), mean (SD)	58.6 (10.0)	61.2 (8.1)	0.26[1]
Median (IQR)	59.5 (50.8-65.3)	63 (57.0-67.0)	
Sex, female, *n* (%)	20 (58.8)	12 (38.7)	0.14[2]
Population group, *n* (%)			1.00[2]
Jews	10 (29.4)	10 (32.3)	
Arabs	24 (70.6)	21 (67.7)	
BMI (kg/m^2), mean (SD)	31.4 (3.8)	32.8 (5.2)	0.22[1]
Median (IQR)	31.2 (28.9-33.6)	31.7 (29.3-36.1)	
Diabetes duration (yr), median (IQR)	9.5 (4.8-12.5)	11 (7-17)	0.13[3]
eGFR (mL/min/1.73 m^2 body surface area) at baseline, median	90.4 (74.9-105.5)	93.3 (76.0-100.3)	0.78[3]
HbA1C (%), median (IQR)	7.4 (6.6-9.1)	7.4 (6.6-9.4)	0.93[3]
Metformin, *n* (%)	30 (88.2)	26 (83.9)	0.73[4]
DPP-4, *n* (%)	10 (29.4)	8 (25.8)	0.79[2]
Sulfonylurea, *n* (%)	5 (14.7)	2 (6.5)	0.43[4]
SGLT2 inhibitors, *n* (%)	11 (32.4)	7 (22.6)	0.42[2]
GLP-1 agonists, *n* (%)	5 (14.7)	7 (22.6)	0.53[2]
Basal insulin, *n* (%)	8 (23.5)	10 (32.3)	0.58[2]
Prandial insulin, *n* (%)	3 (8.8)	3 (9.7)	1.00[4]
Current smoking, *n* (%)	4 (11.8)	0 (0)	0.12[4]
No albuminuria, *n* (%)	21 (61.8)	24 (77.4)	0.19[2]
			2-sided
			0.14[2]
			1-sided
Albuminuria < 30 mg/g, *n* (%)	13 (38.2)	7 (22.6)	0.145[3]
			2-sided
Albuminuria 30-300 mg/g, *n* (%)	8 (23.5)	6 (19.4)	0.073[3]
Albuminuria > 300 mg/g, *n* (%)	5 (14.7)	1 (3.2)	1-sided
Retinopathy, *n* (%)	4 (16.7)	3 (13.6)	1.00[4]
NLR_{max} median (IQR)	2.7 (1.7-8.5)	9.2 (5.7-15.4)	< 0.001[3]
CRP (mg/L) at admission, median (IQR)	15.4 (5.1-80.9)	122.1 (70.8-177.2)	< 0.001[3]
CRP_{max} (mg/L) at hospitalization, median (IQR)	15.4 (5.1-86.2)	142.3 (87.1-205.1)	< 0.001[3]
eGFR (mL/min/1.73 m^2 body surface area) at hospitalization, median (IQR)	91.2 (76.0-105.0)	93.3 (76.5-100.3)	0.90[3]
Albumin (g/dL), mean (SD)	3.8 (0.4)	3.3 (0.4)	< 0.001[1]
Median (IQR)	3.9 (3.6-4.1)	3.3 (3.1-3.5)	

[1]Independent sample *t*-test.
[2]Chi-square test.
[3]Mann-Whitney test.
[4]Fisher's exact test.
COVID-19: Coronavirus disease 2019; IQR: Interquartile range; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; eGFR: Estimated glomerular

filtration rate; DPP-4: Dipeptidyl-peptidase 4; SGLT2: Sodium/glucose cotransporter 2; GLP-1: Glucagon-like peptide 1; NLR_{max}: Maximum neutrophil:lymphocyte ratio; CRP: C-reactive protein; CRP_{max}: Maximum C-reactive protein.

Table 4 Multivariable logistic regression analysis performed to determine the risk factors for severe coronavirus disease 2019 infection (*n* = 65)

		OR	95%CI	*P* value
Model 1	Sex (female *vs* male)	0.30	0.10-0.93	0.038
Baseline characteristics	Age (yr)	1.03	0.98-1.09	0.28
R-square: 14.4%	BMI (kg/m^2)	1.12	0.99-1.28	0.073
Model 2	Sex (female *vs* male)	0.57	0.15-2.16	0.41
Baseline characteristics	Age (yr)	1.02	0.94-1.11	0.62
R-square: 34.4%	BMI (kg/m^2)	1.14	0.96-1.35	0.13
	HbA1c (%)	0.85	0.59-1.23	0.39
	Baseline eGFR (mL/min/ 1.73 m^2 of body surface area)	1.02	0.98-1.07	0.28
	NLR_{max}	1.18	1.05-1.33	0.007
Model 3	Sex (female *vs* male)	0.42	0.09-1.94	0.27
Baseline characteristics	Age (yr)	1.08	0.97-1.20	0.18
R-square = 46.9%	BMI (kg/m^2)	1.24	1.01-1.53	0.040
	HbA1c (%)	1.04	0.67-1.61	0.89
	Baseline eGFR (mL/min/1.73 m^2 of body surface area)	1.04	0.99-1.09	0.12
	NLR_{max}	1.20	1.06-1.37	0.005
	Albuminuria	0.09	0.01-0.62	0.015

OR: Odds ratio; CI: Confidence intervals; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; eGFR: Estimated glomerular filtration rate; NLR_{max}: Maximum neutrophil:lymphocyte ratio; CRP: C-reactive protein; IQR: Interquartile range.

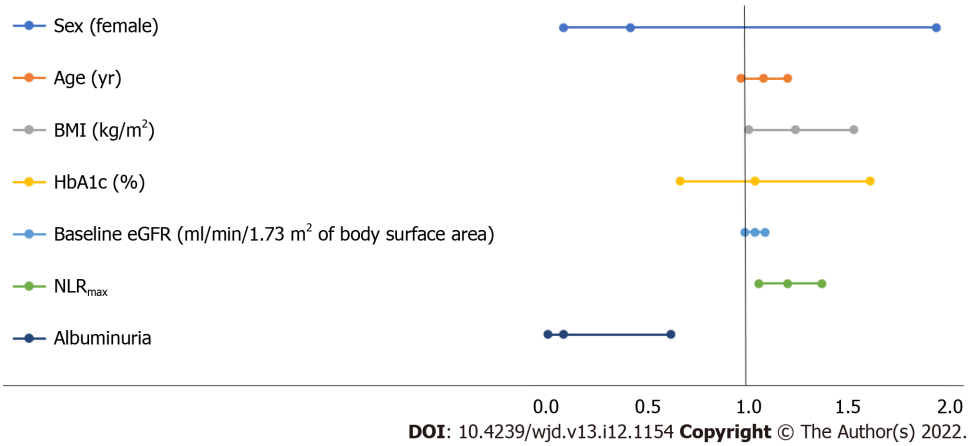


Figure 2 Multivariable logistic regression Model 3 as shown in Table 4. The dependent variable is the severity of coronavirus disease 2019 pneumonia, while the following parameters are independent variables: age, sex, body mass index, last estimated glomerular filtration rate measured before hospitalization, glycosylated hemoglobin, maximum neutrophil:lymphocyte ratio, and albuminuria before hospitalization. BMI: Body mass index; eGFR: Estimated glomerular filtration rate; HbA1c: Glycosylated hemoglobin; NLR_{max}: Maximum neutrophil:lymphocyte ratio.

antiglycemic therapies, as predictors of severe COVID-19 pneumonia. Similarly to previous publications, we identified factors that were significantly associated with increased severity of COVID-19 pneumonia[3-5], including higher BMI (OR 1.24, 95%CI: 1.01-1.53, *P* = 0.04) and NLR_{max} (OR 1.2, 95%CI:

Table 5 Multivariable logistic regression analysis performed to determine the risk factors for bacterial infection (*n* = 144)

		OR	95%CI	*P* value
Model 1	Sex (female *vs* male)	1.88	0.70-5.05	0.21
Baseline characteristics	Age (yr)	0.98	0.93-1.02	0.34
R-square: 2.8%	BMI (kg/m^2)	0.95	0.88-1.04	0.27
Model 2	Sex (female *vs* male)	7.23	1.57-33.23	0.011
Baseline characteristics	Age (yr)	1.00	0.92-1.09	0.97
R-square: 54.0%	BMI (kg/m^2)	0.94	0.84-1.05	0.26
	HbA1c (%)	1.19	0.88-1.60	0.25
	Baseline eGFR (mL/min/1.73 m^2 of body surface area)	1.05	1.01-1.08	0.013
	NLR_{max}	1.36	1.21-1.54	< 0.001
Model 3	Sex (female *vs* male)	7.26	1.58-33.44	0.011
Baseline characteristics	BMI (kg/m^2)	0.94	0.84-1.05	0.26
R-square: 54.0%	Age (yr)	1.00	0.92-1.09	0.98
	HbA1c (%)	1.19	0.87-1.61	0.28
	Baseline eGFR (mL/min/1.73 m^2 of body surface area)	1.05	1.01-1.08	0.012
	NLR_{max}	1.37	1.21-1.55	< 0.001
	Albuminuria	1.12	0.32-3.96	0.86

OR: Odds ratio; CI: Confidence intervals; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; eGFR: Estimated glomerular filtration rate; NLR_{max}: Maximum neutrophil:lymphocyte ratio.

1.06-1.37, P = 0.005), the latter reflecting the immune modulation caused by COVID-19 viremia. Surprisingly, the presence of moderate-range albuminuria before hospitalization was associated with reduced risk for severe COVID-19 pneumonia (OR 0.09, 95%CI: 0.01-0.62, P = 0.015).

Several oral antiglycemic therapies may yield protective anti-COVID-19 properties due to their pleotropic effects. Given that DPP-4 is thought to be one of the COVID-19 receptors and DPP-4 inhibitors have anti-inflammatory activity[15], these medications were indeed associated with a better clinical outcome in COVID-19 patients[8,16]. GLP-1 receptor agonists have several immune modulation activities, including inhibition of nuclear factor-κB[5,17]. SGLT2 inhibition decreases the mRNA expression levels of some cytokines and chemokines, such as tumor necrosis factor, interleukin-6, and monocyte chemoattractant protein 1[14]. Metformin treatment reduces the circulating levels of inflammatory biomarkers in diabetics, and was associated with significantly lower in-hospital mortality in a retrospective study of COVID-19 patients[5,18,19]. Despite the above properties, in the current study we did not detect a protective effect of any class of antiglycemic medication. It is possible that the current cohort was underpowered to detect such differences.

The protective association of baseline moderate-range albuminuria with severe COVID-19 pneumonia was unexpected and counterintuitive, given the well-established role of CKD in enhancing severity of the disease. This association was not observed in patients suffering from bacterial infection, suggesting a specific protective effect against COVID-19, as we cannot draw any conclusions about other viral pathogens in the current cohort. To our knowledge, baseline moderate-range albuminuria with eGFR > 60 mL/min has not been described as conferring such an advantage as demonstrated here, and we cannot draw any conclusions about other viral pathogens in the current cohort.

How can we reconcile this protective association between baseline albuminuria and reduced risk for severe COVID-19 pneumonia?

Post-mortem findings in the lungs of people with fatal COVID-19 demonstrated diffuse alveolar damage and inflammatory cell infiltration[20]. The inflammatory response in patients with severe COVID-19 pneumonia is impaired. Specifically, it has been demonstrated that interferon (IFN) type I response is disrupted with low IFNα activity in the blood, indicating high blood viral load and an impaired inflammatory response[21]. Despite IFN's protective role in COVID-19 infection, IFN can lead to proteinuria. The role of activation of type I IFN signaling in mediating proteinuria is well-known. Podocytes respond to toll-like receptor ligand-like polyinosinic:polycytidylic acid [poly (I:C)] that simulates viral infection, by releasing pro-inflammatory cytokines and activation of type I IFN signaling. IFN signaling enhances podocyte B7-1 expression and actin remodeling *in vitro* and leads to

transient proteinuria *in vivo*. Interestingly, mice treated with a type I IFN receptor-blocking antibody were protected from lipopolysaccharide-induced proteinuria[22]. Therefore, we hypothesize that the presence of moderate-range albuminuria may represent intact type 1 IFN signaling, and in the case of COVID-19 infection, such intact IFN signaling can confer protection from severe COVID-19 pneumonia. We emphasize that moderate-range albuminuria has a protective association in the context of patients with mild CKD (eGFR > 60 mL/min), who constitute the majority of participants in our cohort. Notwithstanding, it was recently demonstrated that patients with eGFR < 60 mL/min or severe albuminuria are at risk for severe COVID-19 infection[23], probably due to advanced CKD contributing to impaired immune function.

A similarly counterintuitive association was recently demonstrated in a cohort of inflammatory bowel disease (IBD) patients. Severe sequelae of COVID-19 were lower in IBD patients compared to matched non-IBD controls, suggesting that baseline immune activity may modulate the progression of COVID-19 pneumonia[24]. In addition, the unexpected finding with regard to moderate-range albuminuria and protection from severe COVID-19 pneumonia might be the result of confounding by as yet unidentified factors or collider bias.

As with all observational studies, our study has limitations. We did not have information regarding other co-morbidities such as liver disease, respiratory disease, alcohol abuse, cognitive impairment, *etc.*, which can potentially serve as confounding factors. Residual confounding might also have resulted from the use of only several measurements to identify baseline characteristics. We did not have data about urine albumin:creatinine during hospitalization; therefore, the contribution of this variable was not included in the final model. In addition, we did not have baseline clinical data for patients who did not survive COVID-19 infection, as described in the methods relating to patient enrollment. Of note, the sample size was small, but was adequate to demonstrate statistical significance of the protective association between moderate-range albuminuria and severe COVID-19 pneumonia.

CONCLUSION

The presence of moderate-range albuminuria in patients with diabetes suffering from COVID-19 pneumonia may represent an intact IFN type 1 response that may translate to protection against severe disease. Further studies are needed to explore whether this association is also relevant to other viral infections and characterize the pathogenesis relating to immunomodulation, which may indicate a biomarker for patients at reduced risk for COVID-19-related deterioration that may translate to therapeutic interventions.

ARTICLE HIGHLIGHTS

Research background

Several risk factors for severe coronavirus disease 2019 (COVID-19) disease have been described, including: Advanced age, male sex, smoking history, and underlying chronic diseases such as cardiovascular disease, diabetes mellitus, obesity, underweight, and chronic kidney disease (CKD). This case-control study was conducted to identify risk factors for severe COVID-19 pneumonia in patients with type 2 diabetic mellitus hospitalized at the Galilee Medical Center (Nahariya, Israel).

Research motivation

We aimed to characterize differences between hospitalized diabetic patients with *vs* patients without COVID-19, and parameters associated with COVID-19 severity for prediction.

Research objectives

Similar to previous reports, multivariable logistic regression showed higher body mass index (BMI) and neutrophil:lymphocyte ratio (NLR) were significantly associated with severe COVID-19. Surprisingly, pre-hospitalization albuminuria, mostly moderate-range (albumin:creatinine 30-300 mg/g), was associated with reduced risk for severe COVID-19 pneumonia. The counterintuitive protective association in patients with stage II CKD was not described before. Given the causative association between type I interferon (IFN) signaling and proteinuria, we hypothesize that the presence of moderate-range albuminuria may represent an intact type I IFN signaling, which confers protection from severe COVID-19 pneumonia and its complications.

Research methods

This case-control study included 209 patients with type 2 diabetic mellitus hospitalized at the Galilee Medical Center (Nahariya, Israel) and recruited between September 2020 and May 2021, 65 patients with COVID-19 infection in dedicated wards and 144 COVID-19-negative patients in internal medicine

wards hospitalized due to other reasons. Clinical parameters – including age, type of antiglycemic medications, presence of retinopathy, smoking history, BMI, glycosylated hemoglobin, maximum NLR (NLR_{max}), C-reactive protein (CRP_{max}), estimated glomerular filtration rate (eGFR), and albumin (blood and urine) - were compared between the two primary patient groups, and then between COVID-19-negative patients hospitalized due to infectious *vs* non-infectious disease. Finally, we explored which parameters were associated with severe COVID-19 pneumonia.

Research results

COVID-19-negative patients were older and had longer duration of diabetes, lower eGFR, higher albumin, lower CRP, greater smoking history, and more baseline albuminuria at admission. 70% of COVID-19 patients with albuminuria had moderate-range albuminuria. Most of the patients with albuminuria had CKD II. Oral antiglycemic therapies were not significantly different between the two groups. As previously reported, multivariable logistic regression showed higher BMI and higher NLR were significantly associated with severe COVID-19. Surprisingly, pre-hospitalization albuminuria, mostly moderate-range, was associated with reduced risk for severe COVID-19 pneumonia. This protective association was specific to COVID-19 infection and was not observed in bacterial infections.

Research conclusions

Moderate-range albuminuria in COVID-19-positive diabetic patients with CKD II is associated with less severe COVID-19. We hypothesize that this counterintuitive association may represent intact IFN signaling that on the one hand can lead to harmful proteinuria *via* podocyte injury, and on the other hand can serve as a protective cytokine with the potential to mitigate COVID-19 infection and complications. Given the importance of intact type I IFN response in controlling COVID-19, we suggest that moderate-range albuminuria in diabetic patients with mild CKD may serve as a biomarker for intact IFN signaling and therefore is associated with reduced risk for severe COVID-19 pneumonia.

Research perspectives

Further studies should explore the potential role of albuminuria in the presence of mild CKD as a biomarker for reduced risk of COVID-19-related deterioration that may translate to therapeutic interventions.

ACKNOWLEDGEMENTS

Ossie Sharon contributed to the editing of this paper.

FOOTNOTES

Author contributions: Bashkin A was the guarantor and designed the study, and was responsible for conceptualization, project administration, supervision, methodology, writing, review and editing; Shehadeh M and Shbita L were responsible for data curation and participated in formal analysis; Namoura K, Haiek R, Boulos Y, and Kuyantseva E participated in data curation; Yakir O was responsible for formal analysis; Kruzel-Davila E was responsible for methodology, formal analysis, writing, review and editing.

Institutional review board statement: The study was reviewed and approved by the Helsinki Committee of the Galilee Medical Center.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement checklist of items, and the manuscript was prepared accordingly.

Country/Territory of origin: Israel

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S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

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World Journal of Diabetes

Submit a Manuscript: https://www.f6publishing.com

World J Diabetes 2022 December 15; 13(12): 1168-1183

DOI: 10.4239/wjd.v13.i12.1168

ISSN 1948-9358 (online)

SYSTEMATIC REVIEWS

Comparison of gliclazide *vs* linagliptin on hypoglycemia and cardiovascular events in type 2 diabetes mellitus: A systematic review

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Specialty type: Medicine, research and experimental

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Feng JF, China; Gluvic Z, Serbia; Ma JH, China; Su G, China

Received: July 20, 2022
Peer-review started: July 20, 2022
First decision: September 4, 2022
Revised: September 20, 2022
Accepted: October 27, 2022
Article in press: October 27, 2022
Published online: December 15, 2022

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Abstract

BACKGROUND

Cardiovascular outcome trials have demonstrated cardiovascular safety of glimepiride (a sulfonylureas) against dipeptidyl peptidase-4 inhibitor linagliptin. Gliclazide (another newer sulfonylureas) has shown similar glycemic efficacy and 50% decreased risk of hypoglycemia compared to glimepiride.

AIM

Considering the absence of cardiovascular outcome trials for gliclazide, we decided to conduct a systematic review of the literature to assess the cardiovascular (CV) safety by assessing the risk for major adverse CV events and hypoglycemia risk of gliclazide *vs* linagliptin in patients with type 2 diabetes (T2D).

METHODS

This systematic review followed the current Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines to analyze all the clinical studies published from 2008 that compared the two drugs in patients with T2D with no risk of CV disease (CVD). We included only evidence designated high quality by the Oxford Center for Evidence-based Medicine-Levels of Evidence.

RESULTS

Eight clinical studies were included in the narrative descriptive analysis

(gliclazide: 5 and linagliptin: 3). The CV safety of gliclazide in the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation trial and of linagliptin in the Cardiovascular and Renal Microvascular Outcome Study With Linagliptin (CARMELINA) and CARdiovascular Outcome study of LINAgliptin *vs* glimepiride in patients with T2D (CAROLINA) trials were excluded from the comparative analysis as these trials demonstrated CV and hypoglycemia benefits in patients at high risk of CVD. However, since these are landmark trials, they were discussed in brief to show the CV benefits and low hypoglycemia risk of gliclazide and linagliptin. We did not find any study comparing gliclazide with linagliptin. Hence, direct comparison of their major adverse CV events and hypoglycemia risk could not be carried out. However, the literature meeting the inclusion criteria showed that both drugs were effective in achieving the desired glycemic control and had low major adverse CV events and hypoglycemia risk in adult patients with no history of CVD.

CONCLUSION

Gliclazide can be considered an effective and safe glucose-lowering drug in T2D patients with no established CVD but at high risk of CVD due to their T2D status. Future randomized controlled trials comparing gliclazide with linagliptin or dipeptidyl peptidase-4 inhibitors can confirm these findings.

Key Words: Linagliptin; Gliclazide; Hypoglycemia; Major cardiovascular adverse events; Type 2 diabetes

Core Tip: This systematic review showed the lack of high-quality evidence and head-to head trials comparing the cardiovascular safety and hypoglycemia risk of gliclazide (a sulfonylurea) *vs* linagliptin (dipeptidyl peptidase-4 inhibitor) in adults with type 2 diabetes and no cardiovascular disease. While dipeptidyl peptidase-4 inhibitors have been proven to be cardiovascular neutral, sulfonylureas like gliclazide are commonly prescribed and recommended glucose-lowering drugs in low resource settings. Hence, it is important to establish the cardiovascular safety and hypoglycemia risk of gliclazide *vs* linagliptin to highlight that gliclazide may be a cost-effective yet safe treatment option for patients with type 2 diabetes.

Citation: Mohan V, Wangnoo S, Das S, Dhediya R, Gaurav K. Comparison of gliclazide *vs* linagliptin on hypoglycemia and cardiovascular events in type 2 diabetes mellitus: A systematic review. *World J Diabetes* 2022; 13(12): 1168-1183

URL: https://www.wjgnet.com/1948-9358/full/v13/i12/1168.htm

DOI: https://dx.doi.org/10.4239/wjd.v13.i12.1168

INTRODUCTION

Type 2 diabetes (T2D), characterized by chronic hyperglycemia and impaired insulin secretion, is often associated with disease-related microvascular and macrovascular complications and treatment-related complications like hypoglycemia[1,2]. Consequently, patients with T2D are at an increased risk for cardiovascular (CV) complications and hypoglycemia. Hence, glucose-lowering drugs (GLDs) should not have CV complications and higher hypoglycemic episodes (HE) as adverse effects (AEs) and should ideally provide CV benefits or neutrality[1,2].

Sulfonylureas (SUs) are the most prescribed T2D pharmacotherapy, especially in resource limited settings[3]. Apart from their cost benefit, Sus have an exceptional glycemic efficacy with average glycosylated hemoglobin (HbA1c) reduction by 1%-2%, good safety profile and gastrointestinal tolerability[3]. However, hypoglycemia, weight gain and decreasing efficacy over time are the main concerns with SUs due to their insulinotropic mechanism of action[3-5]. On the other hand, newer oral GLDs like dipeptidyl peptidase-4 (DPP4) inhibitors and sodium-glucose cotransporter-2 (SGLT2) inhibitors provide comparably less glycemic control than SUs (average HbA1c reduction 0.5%-0.8%), are costlier than SUs and often need to be combined with SUs to achieve the required glycemic control[3].

However, since, the time of their inception into T2D treatment regime, SUs have been subjected to criticism for CV safety[3,6]. The CV safety of SUs has been derived from small, inadequately powered randomized controlled trials (RCTs) and observational studies[3]. However, formal cardiovascular outcome trials (CVOTs) are not available for SUs[3,6].

Then, in 2008, the United States Food and Drug Administration mandated the assessment of CV safety of newer GLDs[7]. Hence, large multinational, CVOTs of newer oral GLDs like DPP4 inhibitors[8-12] and SGLT2 inhibitors[13-15] were conducted and showed their CV benefits. DPP4 inhibitors and SGLT2 inhibitors proved to be costly options in resource limited settings because of the chronic disease nature of T2D and because most patients pay from their pocket for the treatment[16,17].

Despite their unquestionable glucose lowering efficacy, current diabetes guidelines no longer favors the use of SUs because of CV safety concerns except when cost is an issue[3,6]. SUs have been recommended as the add-on of choice after metformin for adequate glycemic control in resource limited settings by the World Health Organization (WHO) Guidelines, the Research Society for the Study of Diabetes in India/Endocrine Society of India (RSSDI-ESI) (2020) guidelines from India[18,19], the International Task Force (ITF) Consensus[20] and the International Diabetes Federation (IDF)[21]. The ITF recommends glimepiride and gliclazide modified release (MR) as the SU of choice to be added to metformin, while the IDF gave equal importance to SUs (except glibenclamide/glyburide), a DPP4 inhibitor or an SGLT2 inhibitor[20,21].

The American Diabetes Association (ADA) (2021) guidelines recommend various add-on pharmacotherapies for T2D patients poorly controlled on metformin, including DPP4 inhibitors, SGLT2 inhibitors and SUs[22]. The American Diabetes Association guidelines recommend T2D patients with CV and renal morbidities should ideally be prescribed SGLT2 inhibitors or glucagon-like peptide-1 (GLP-1) agonists as the next oral GLDs after metformin[22]. However, the choice of add-on therapy in patients without CV risk is not clear.

Of the various DPP4 inhibitors used in T2D, landmark linagliptin trials have demonstrated CV safety and safety against HE in T2D patients with a high risk of CV disease (CVD)[8,9]. On the other hand, a landmark non-CVOT trial in patients with high CV risk showed that high intensity gliclazide treatment conferred low CV risk[23].

Many systematic reviews (SRs) and/or meta-analyses (MAs) have assessed the efficacy and safety [hypoglycemia and major adverse cardiovascular events (MACE; CV death, nonfatal myocardial infarction/ischemia/acute coronary syndrome or nonfatal stroke)] of SUs *vs* DPP4 inhibitors with mixed results[24-28]. These SRs and meta-analyses identified a need for RCTs comparing individual SUs with a DPP4 inhibitor. Hence, this SR was carried out to assess the CV safety and hypoglycemia risk of gliclazide *vs* linagliptin in T2D patients, both in monotherapy and as add-on to metformin setting.

MATERIALS AND METHODS

Methodology

The MEDLINE database was searched on September 9, 2021 for records on gliclazide or linagliptin with no filter added. This retrieved 2578 records. An advanced search filter was then applied to filter by English language only, clinical trials, RCT, human studies and adult age (19 + years). These filters retrieved 2054 records. The records were further filtered by applying adverse events of interest: hypoglycemia, low blood sugar, myocardial infarction/myocardial ischemia (MI), transient ischemic attack, CV death and stroke. This retrieved 615 records; 223 duplicates were removed and the remaining 392 records were screened. It was seen that linagliptin records were available from 2008 onwards only. Hence, to standardize the time period for the entire literature search, gliclazide records published before 2008 were removed. The remaining 248 records were assessed for eligibility. After excluding records that did not meet the eligibility criteria as mentioned in Table 1, eight records were included (5 for gliclazide and 3 for linagliptin). The details of the literature search and study selection are outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart (Figure 1). Google Scholar was searched for any additional manuscripts that were missed on MEDLINE. This retrieved no additional records as per study selection criteria.

Two independent reviewers used the current PRISMA guidelines for SRs[29,30] to independently carry out the literature search on the same day. Any conflict in the number of records at identification, screening, eligibility and inclusion were mutually discussed and resolved by consensus. We do note that the protocol for this systematic review has not been published.

Quality of evidence and risk of bias

As shown by the PRISMA flow chart, there were many articles regarding both gliclazide and linagliptin. Hence, we included only high-quality evidence. RCTs were designated the highest quality by the Oxford Center for Evidence-based Medicine-Levels of Evidence[31] followed by a randomized design of any type. Hence, we included only randomized studies. Placebo controlled studies were not included as there were no gliclazide *vs* placebo studies. The main reason for this could be that trials in the initial trajectory of drug development were missed by standardizing the study period from 2008 onwards. Additionally, studies comparing gliclazide or linagliptin with metformin were also not included because both drugs have a known and comparable efficacy and safety profile *vs* metformin.

Table 1 Inclusion and exclusion criteria of the records included in the systematic review

Inclusion criteria	Exclusion criteria
Age 19 yr and < 70 yr; Male and Female; type 2 diabetes	Age below 19 yr or ≥ 70 yr; type 1 diabetes; no diabetes
Human studies: Any race, ethnicity	Clinical trials evaluating gliclazide or linagliptin in patients with specific comorbidites including CVD[1]
Randomized clinical trials on safety of: -Gliclazide monotherapy versus linagliptin monotherapy -Gliclazide + metformin versus linagliptin + metformin	Review articles, systematic reviews and meta-analysis, network meta-analysis, pooled analysis of trials, case studies, non-randomized trials
Randomized clinical trials on safety of: -Gliclazide versus DPP4 inhibitors -Linagliptin versus sulfonylureas	Pharmacokinetic, pharmacodynamic and bioequivalence study; retrospective chart review; observational real-world study; case study; trials studying mechanism of action of gliclazide or linagliptin; literature reporting only study design; trial summaries and implications; animal studies; preclinical studies
Randomized clinical trials on gliclazide or linagliptin monotherapy evaluating the following outcomes:	Clinical trials evaluating gliclazide or linagliptin versus PBO
-Hypoglycemia or low blood sugar	Clinical trials evaluating gliclazide or linagliptin in combination with other GLDs except metformin
-Occurrence of 3 point major adverse cardiovascular events (3P-MACE): Cardiovascular death, nonfatal myocardial infarction/ischemia/acute coronary syndrome, or nonfatal stroke (transient ischemic attack included)	Clinical trials evaluating gliclazide or linagliptin versus other GLDs except: (1) DPP4 inhibitors for gliclazide; and (2)sulfonylureas for linagliptin
	Clinical trials evaluating other glycemic, cardiac, cardiovascular outcomes than those of interest; other outcomes (*e.g.*, microvascular complications)

[1]History of myocardial infarction, stroke, unstable angina, transient ischemic attack, percutaneous coronary intervention for coronary occlusion or coronary artery bypass graft.

Note: Efficacy was synthesized from the gliclazide and linagliptin studies that met the inclusion criteria. CVD: Cardiovascular disease; DPP4: Dipeptidyl peptidase-4; GLD: Glucose-lowering drugs; PBO: Placebo.

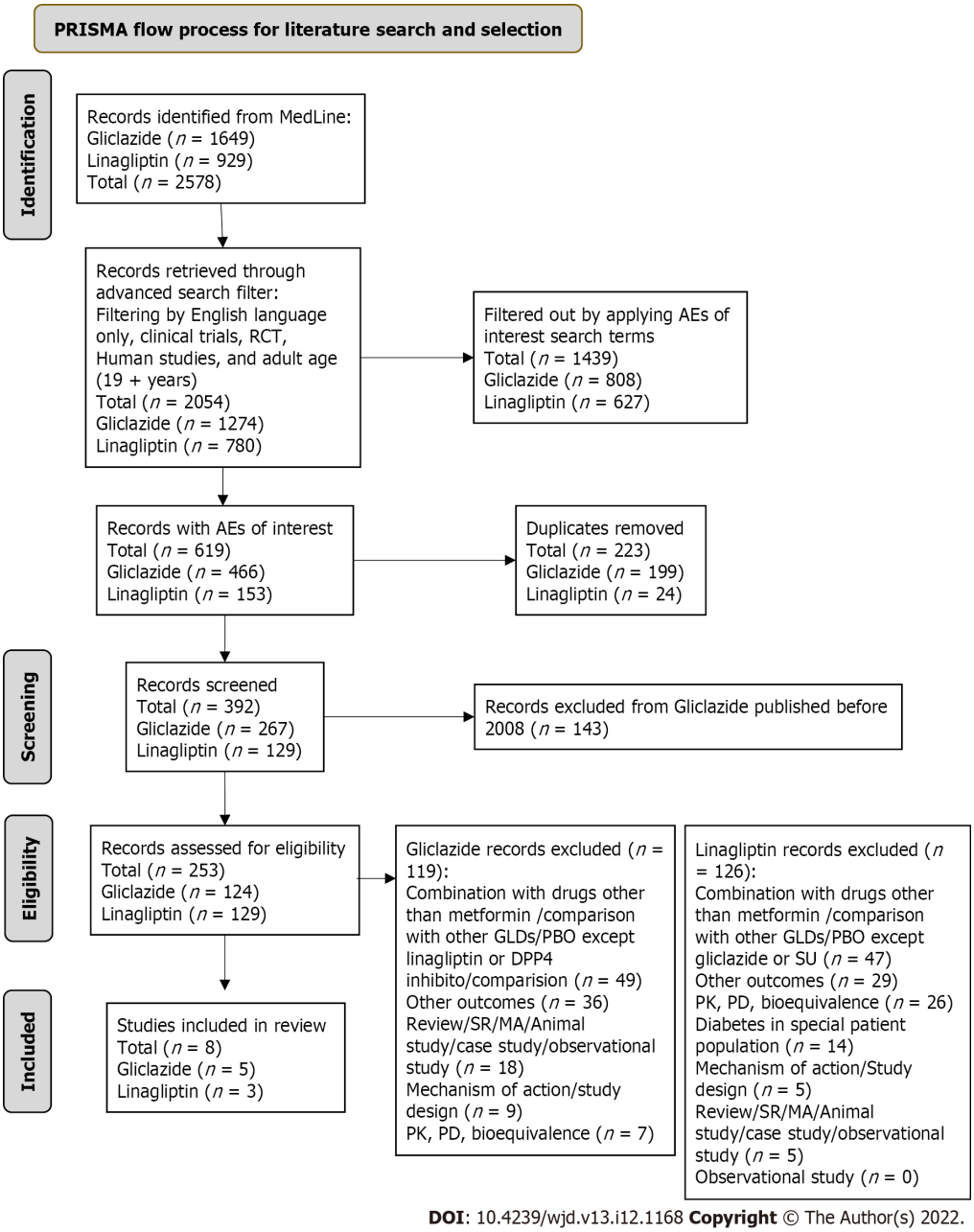
Further, risk of bias assessment was independently carried out by two researchers who assessed the scientific quality of the records using the Cochrane Collaboration's tool for risk of bias assessment[32]. The Cochrane Risk of Bias tool assesses seven domains of bias and stratifies the risk of bias as low, high and unclear risk. Discrepancies between reviewers at any stage were resolved by discussion and consensus.

All the studies clearly defined and reported the outcomes of interest (hypoglycemia and MACE) and clearly mentioned all the CVDs that were assessed as exclusion criteria. Only one gliclazide trial[33] did not have any CVD as an exclusion criteria. The trials clearly explained the randomization schedule and were largely double-blind studies. The number of participants for which the outcomes of interest were reported was clearly stated.

However, most studies were not designed to report the outcome of interest (hypoglycemia and MACE) as their main primary and/or secondary endpoint. These outcomes of interest were primarily reported as AEs or safety endpoints.

Statistical analysis

The systematic literature search (Figure 1) did not retrieve any head-to-head trials comparing gliclazide ± metformin with linagliptin ± metformin. Hence, direct comparison of their outcomes was not possible. The gliclazide and linagliptin trials that met the inclusion criteria could not be compared to reach a statistical analysis due to various reasons. The studies captured for the two drugs were heterogeneous with respect to study design and duration, the outcomes of interest being evaluated as primary or secondary or safety (as AE) endpoints or as incident findings, definition of outcomes [*e.g.*, definition of hypoglycemia-cut off blood glucose (BG) level] and the statistical method used for analysis. The study population of the various studies differed in age, ethnicity and patient profile (*e.g.*, treatment naïve or after failure of SU). Hence, a meta-analysis or a network meta-analysis could not be carried out. Therefore, key outcomes were described in a narrative manner for each drug separately, with due consideration given to the PRISMA checklist[29].



DOI: 10.4239/wjd.v13.i12.1168

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow chart of literature search and selection. AE: Adverse event; DPP4: Dipeptidyl peptidase 4; GLD: Glucose-lowering drug; MA: Meta-analysis; PBO: Placebo; PD: Pharmacodynamic; PK: Pharmacokinetic; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCT: Randomized controlled trials; SR: Systematic review; SU: Sulfonylurea.

RESULTS

Gliclazide studies

This section aimed to include RCTs that compared gliclazide *vs* linagliptin or a DPP4 inhibitor in a monotherapy setting or compared gliclazide as an add-on to metformin *vs* linagliptin/DPP4 inhibitor as add-on to metformin.

Gliclazide *vs* linagliptin or DPP4 inhibitors: There were no records comparing gliclazide with linagliptin. One study compared gliclazide with vildagliptin, a DPP4 inhibitor[34] (Table 2). Foley *et al* [34] compared the efficacy and safety of 2 years of monotherapy with vildagliptin *vs* gliclazide in 1092 drug-naïve patients with T2D having HbA1c of 7.5%-11.0%. In this vildagliptin non-inferiority trial, the vildagliptin group had a lower incidence of grade 1 hypoglycemia than the gliclazide group (0.7% *vs* 1.7%).

Two patients in the gliclazide group and none in the vildagliptin group had ≥ 2 HEs[34]. Though the baseline HbA1c values were slightly higher in the group treated with gliclazide *vs* the vildagliptin group (HbA1c of 8.7% ± 0.1% *vs* 8.5% ± 0.1%), the mean HbA1c reduction from baseline to week 104 was

Table 2 Gliclazide *vs* dipeptidyl peptidase-4 inhibitor /linagliptin *vs* sulfonylurea

Ref.	Primary study objective	Study design	Study population	CVD excluded	Number of participants	Study duration	Endpoint (hypoglycemia)	Hypoglycemia definition	Hypoglycemia results	Endpoint (MACE)	MACE definition	MACE results
Gliclazide *vs* DPP4 inhibitor (vildagliptin)												
Foley *et al* [34], 2009	Compare the efficacy and safety of vildagliptin *vs* gliclazide	Randomized, multicenter, double-blind, active-controlled study	Drug-naïve patients with T2D, HbA1c of 7.5%-11.0%	CHF NYHA class III or IV, ECG abnormalities	1092	104 wk	AEs were safety endpoints	Grade 1 hypoglycemic events per week: symptoms suggestive of low blood glucose confirmed by SMBG measurement of < 3.1 mmol/L plasma glucose equivalent not requiring the assistance of another party; Grade 2 hypoglycemic event (requiring the assistance of another party) or if there were 3 or more asymptomatic glucose values < 3.1 mmol/L per week	Grade 1 hypoglycemia: 4 patients (0.7%) in the vildagliptin group and 14 (1.7%) in the gliclazide group. ≥ 2 HEs: 2 patients in the gliclazide group and none in vildagliptin group No grade 2 HEs in either group	-	-	-
Gliclazide + metformin *vs* DPP4 inhibitor (vildagliptin) + metformin												
Vianna *et al* [35], 2018 (Part of BoneGlic Trial)	Compare the effects on glycemic variability and bone metabolism	Single center, randomized, controlled, open-label (blinded to the observer)	Postmenopausal Brazilian women with T2D and treated with a stable metformin dose for ≤ 3 mo	CV complications	56 (42 randomized)	2-wk pre-randomization period followed by 24 wk	As AE	Major hypoglycemia: glucagon, carbohydrates administration by another person or other resuscitative measures; minor hypoglycemia: BG ≤ 3.9 mmol/L with or without typical symptoms or hypoglycemia symptoms without BG test	No differences from baseline in time to hypoglycemia (% of time ≤ 3.9 mmol/L) No major hypoglycemia Minor hypoglycemia events: 7 in the gliclazide; 2 in the vildagliptin group (P = 0.062)	As SAE		Vildagliptin: 1 hemorrhagic stroke gliclazide MR group: 1 death due to AMI, the investigator did not consider the SAEs to be related to the study medications
Hassanein *et al*[36], 2014 (STEADFAST study)	HE during Ramadan	Multiregional, randomized double-blind	Patients fasting during Ramadan	CHF (NYHA class III or IV); other significant CV history within 6 mo	557	4-wk Ramadan period	Primary	Hypoglycemia: Low BG symptoms with or without confirmatory, SMBG measurement < 3.9 mmol/L; PGE or asymptomatic SMBG < 3.9 mmol/L PGE; confirmed hypoglycemia: symptomatic/asymptomatic SMBG measurement < 3.9 mmol/L; PGE and severe HE	Confirmed and/or severe HE during Ramadan: vildagliptin *vs* glicalzide was 3.0% *vs* 7.0% (P = 0.039; one-sided test); HEs: vildagliptin *vs* gliclazide was	-	-	-

								requiring assistance from another party irrespective of whether SMBG value was available or not	6.0% and 8.7% (P = 0.173)			
Filozof and Gautier[37], 2010	Demonstrate non-inferiority of vildagliptin compared with gliclazide, as an add-on therapy	Randomized, double-blind, active-controlled	T2D uncontrolled with metformin	Serious cardiac conditions (torsades de pointes, sustained and clinically relevant VT or VF, PCI ≤ 3 mo, MI, CABG, unstable angina, or stroke ≤ 6 mo and CHF requiring pharma-cological treatment, 2nd- or 3rd-degree AV block or prolonged QTc)	1007	52 wk	AE	Symptoms suggestive of hypoglycemia and confirmed by SMBG < 3.1 mmol/L	HE vildagliptin *vs* gliclazide (6 *vs* 11 events)	-	-	-

AE: Adverse event; AMI: Acute myocardial infarction; AV: Atrioventricular; BG: Blood glucose; CABG: Coronary artery bypass surgery; CHF: Congestive heart failure; CV: Cardiovascular; CVD: Cardiovascular disease; DPP4: Dipeptidyl peptidase-4; ECG: Electrocardiogram; HbA1c: Glycated hemoglobin: HE: Hypoglycemia event/episode; MACE: Major adverse cardiovascular event; MI: Myocardial infarction; MR: Modified release; NYHA: New York Heart Association; PCI: Percutaneous coronary intervention; PGE: Plasma glucose equivalent; SAE: Serious adverse event; SMBG: Self-monitored blood glucose; T2D: Type 2 diabetes; VF: Ventricular fibrillation; VT: Ventricular tachycardia.

-0.5% and -0.6% in the vildagliptin *vs* gliclazide group[34]. The study could not show the non-inferiority of the DPP4 inhibitor over gliclazide.

Gliclazide + metformin *vs* linagliptin/DPP4 inhibitors + metformin: There were no records comparing gliclazide + metformin with linagliptin + metformin. Vianna *et al*[35] compared the glycemic variability of gliclazide MR and vildagliptin and their effect on bone metabolism. This study was the single center part of the BoneGlic Trial, which reported hypoglycemia and MACE as AEs in 42 postmenopausal Brazilian women with T2D and treated with a stable metformin dose for ≤ 3 mo. The study found no difference in time to hypoglycemia and the number of HEs in both the groups (P = 0.062). The investigator did not consider MACE events (Table 2) to be related to study drugs.

The study also found that the gliclazide MR group had a significantly longer time within the target BG range [> 3.9 mmol/L and ≤ 10.0 mmol/L (> 70.27 mg/dL and ≤ 180.18 mg/dL)] and a significantly lower percentage of time with BG > 10 mmol/L (180.18 mg/dL) (P = 0.038 and P = 0.029). In comparison, time within the target BG was insignificantly increased and percentage of time with BG > 10 mmol/L (180.18 mg/dL) was insignificantly lower in the vildagliptin group (P = 0.111 and P = 0.133, respectively). However there were no differences between gliclazide and the DPP4 inhibitor for both the parameters[35].

The STEADFAST study conducted on 557 T2D patients fasting during the holy month of Ramadan found that both gliclazide and vildagliptin as add-on therapy was safe[36]. However, confirmed and/or severe HEs during Ramadan were significantly higher (Table 2) in the glicalzide group[36]. The HEs observed with gliclazide were lower than reported from observational studies. The authors of the

STEADFAST study concluded that HEs with gliclazide could be avoided through frequent patient-physician contacts and Ramadan-focused advice[36].

A vildagliptin non-inferiority trial in patients with T2D uncontrolled with metformin demonstrated that as an add-on to metformin, vildagliptin was non-inferior to gliclazide in achieving glycemic control (95% confidence interval: 0.11%-0.20%)[37]. However, more patients in the vildagliptin group discontinued treatment due to an unsatisfactory effect compared with the gliclazide group (n = 22 *vs* 13, respectively). HEs were lower in the vildagliptin group *vs* the gliclazide group (6 events *vs* 11 events)[37].

All three trials[35-37] comparing gliclazide + metformin with DPP4 inhibitor + metformin described in this section were specific to a patient population (post-menopausal women) or in special situation (fasting during Ramadan). Therefore, these trials did not meet the strict inclusion criteria of this narrative synthesis. They were included because there were no other trials retrieved that compared gliclazide with a DPP4 inhibitor as an add-on therapy. The results on these trials may have been influenced by the patient population or the fasting state of the patients.

Linagliptin studies

This section aimed to include randomized trials that compared linagliptin *vs* gliclazide/SU in a monotherapy setting or compared linagliptin as add-on to metformin *vs* gliclazide/SU as add-on to metformin.

Linagliptin *vs* gliclazide or SUs: There were no studies comparing linagliptin with gliclazide or another SU. The landmark "CARdiovascular Outcome study of LINAgliptin *vs* glimepiride in patients with type 2 diabetes" (CAROLINA)[9] trial and studies[38,39] trial did not meet the inclusion criteria of the narrative synthesis as the study primarily focused on the cardiac and renal patient population. Therefore, other studies[38,39] analyzing the outcomes of interest from the CAROLINA trial were also not included in the narrative synthesis. However, this non-inferiority of linagliptin to glimepiride trial merits discussion as it compared linagliptin with an SU, glimepiride. The trial is covered under the excluded trial section.

However, a study by Barnett *et al*[40] in "metformin contraindicated" T2D patients compared linagliptin 5 mg once daily with placebo for 18 wk and then compared linagliptin with glimepiride after weeks 18 for 34 wk. The study defined hypoglycemia according to the 2005 American Diabetes Association guidelines[41]. The linagliptin group experienced less hypoglycemia [≤ 70 mg/dL (≤ 3.9 mmol/L)] (2.2% *vs* 7.8%) and clinical event committee confirmed CV events (0.7% *vs* 1.6%) than the glimepiride group[40]. However, the difference did not reach clinical significance and more patients in the linagliptin group discontinued treatment due to an AE.

Linagliptin + metformin *vs* gliclazide/SU + metformin: The literature search did not retrieve any linagliptin + metformin *vs* gliclazide/SU + metformin studies meeting the inclusion criteria.

Gliclazide/linagliptin ± metformin

The literature search did not retrieve any gliclazide *vs* placebo studies meeting the inclusion criteria. The main reason for this could be that trials in the initial trajectory of drug development were missed by standardizing the study period from 2008 onwards. Also, there were no trials comparing gliclazide ± metformin with linagliptin ± metformin. Hence, this section aimed to include trials evaluating gliclazide alone or gliclazide + metformin without a comparator and linagliptin alone or linagliptin + metformin without a comparator. These trials were then assessed separately to see if the outcomes of interest could be compared.

Gliclazide ± metformin: Only one trial met the inclusion criteria and is detailed in Table 3. The multicenter, randomized, parallel-group "Diamicron MR in NIDDM: Assessing Management and Improving Control" (DINAMIC 1)[33] trial compared the efficacy, tolerability and acceptability of gliclazide MR for T2D management in the self-monitoring of BG (SMBG) *vs* non SMBG group. HEs were reported as a safety outcome and were classified as follows: Grade 1, suspected mild hypoglycemia; grade 2, suspected moderate hypoglycemia; grade 3, suspected severe hypoglycemia with need of third-party assistance; and grade 4, suspected severe hypoglycemia with need of medical assistance. In 610 T2D patients (aged 40-80 years) followed up for 6 mo, 8.7% patients in the SMBG group had a total of 51 HEs and 7.0% of patients in the non-SMBG group had a total of 66 HEs. There were no severe (grade 3 or 4) HEs in any group.

Symptoms suggestive of nocturnal hypoglycemia were experienced by 3 and 7 patients in the SMBG *vs* non-SMBG, respectively. Two patients withdrew from the study because of hypoglycemia, and both were in the non-SMBG group. The study highlighted the importance of SMBG in T2D management.

Linagliptin ± metformin: Only one trial met the inclusion criteria and is detailed in Table 3. This study compared linagliptin + metformin with only linagliptin and hence was included. Ross *et al*[42] conducted a randomized study to evaluate the efficacy and safety of initial treatment with linagliptin/metformin combination in newly diagnosed T2D patients with marked hyperglycemia. Hypoglycemia occurred in 1.9% of patients in the linagliptin/metformin and 3.2% of patients in the

Table 3 Gliclazide ± metformin and linagliptin ± metformin (no comparator)

Ref./treatment	Primary study objective	Study design	Study population	CVD excluded	Number of participants	Study duration	Endpoint (hypoglycemia)	Hypoglycemia definition	Hypoglycemia results	Endpoint (MACE)	MACE definition	MACE results
Barnett *et al*[33], 2008/DINAMIC 1/Gliclazide	Compare the efficacy, tolerability and acceptability of gliclazide in SMBG *vs* non-SMBG group	Multicenter randomized parallel-group	T2D patients managed on diet alone	Not mentioned	610	6 mo	Safety endpoint (AE)	Grade 1: Suspected mild hypoglycemia	SMBG group: 8.7% patients had 51 HE: symptomatic (27), asymptomatic (11), SMBG-confirmed (11) and non-graded (2)	-	-	-
								Grade 2: Suspected moderate hypoglycemia	Non-SMBG group: 7.0% patients had 66 HE: Symptomatic (66) and non-graded (2). Two HE-related withdrawals			
								Grade 3: Suspected severe hypoglycemia with need of third party assistance	No grade 3 or 4 symptoms			
								Grade 4: Suspected severe hypoglycemia with need of medical assistance	Symptoms suggestive of nocturnal hypoglycemia: SMBG group: 3 and non-SMBG group: 7			
Ross *et al*[42], 2015/Linagliptin/metformin *vs* linagliptin monotherapy	Change from baseline in HbA1c	Randomized, double-blind, active-controlled, parallel group, multinational	Newly diagnosed (≤ 12 mo) T2D and marked hyperglycemia (≥ 8.5 and ≤ 12.0%)	ACS, stroke or TIA < 3 mo	316	24 wk	Safety endpoint (AE)	Severe hypoglycemia: Requiring assistance from another person to administer carbohydrate or other resuscitative action	Linagliptin/metformin: 1.9% of patients and linagliptin: 3.2% of patients no severe hypoglycemia	-	-	No deaths

ACS: Acute coronary syndrome; AE: Adverse event; CVD: Cardiovascular disease; DINAMIC: Diamicron MR in NIDDM: Assessing Management and Improving Control; HbA1c: Glycosylated hemoglobin; HE: Hypoglycemic event; MACE: Major adverse cardiovascular event; SMBG: Self-monitoring of blood glucose; T2D: Type 2 diabetes; TIA: Transient ischemic attack.

linagliptin group. No severe HEs was reported[42]. At week 24, there was a significant reduction in HbA1c from baseline in the linagliptin/metformin *vs* linagliptin group ($P < 0.0001$ for treatment difference)[42]. Target HbA1c of < 7.0% was achieved by 61% of patients in the linagliptin/metformin arm and 40% of patients in the linagliptin arm[42].

Other studies of linagliptin + metformin[43-45] compared the combination with either metformin or with placebo and hence were not included.

Landmark trials not meeting inclusion criteria but requiring special mention

Some landmark and important gliclazide and linagliptin trials were excluded from the narrative synthesis due to the applied exclusion criteria. However, given their importance in the drug trajectory, they require a special mention to obtain a clear picture regarding the HE and MACE AEs associated with gliclazide and linagliptin.

Excluded gliclazide trials

Action in diabetes and vascular disease, Preterax and Diamicron Modified Release Controlled Evaluation trial: Gliclazide studies retrieved during the literature search that reported MACE as an outcome were the "Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation" (ADVANCE)[23] trial and its analyses[46-53]. However, the ADVANCE trial and its analyses were excluded from the narrative synthesis because the ADVANCE trial compared high intensity glucose control (with gliclazide) with standard glucose control (with other SUs). Also, in the high intensity group, those not achieving the targeted HbA1c with highest gliclazide dose were further given metformin, thiazolidinediones, acarbose or insulin as add-on therapy[23]. Comparison studies of gliclazide *vs* other GLDs (except DPP4 inhibitors) and studies analyzing gliclazide in combinations with other GLDs (except metformin) were excluded from the analysis.

Additionally, the ADVANCE trial recruited patients at high CV risk[23,54]. Patients with a history of stroke, MI, unstable angina, transient ischemic attack and coronary or peripheral vascularization met the inclusion criteria for the study[23,54]. Thus, the ADVANCE trial evaluated the MACE outcome in patients at high risk for MACE. However, the ADVANCE trial also recruited patients with no history of CVD but at high risk of MACE as they had T2D for ≥ 10 years or were ≥ 65-years-old.

The primary macrovascular endpoint of the ADVANCE trial was a composite of CV endpoints (death from CV causes, nonfatal MI or nonfatal stroke). Individual CV endpoints were evaluated as secondary endpoints[23,54]. The trial also evaluated microvascular endpoints both as a composite and individual endpoint[23,54]. During the 5-year follow-up there were no significant effects of the type of glucose control on major macrovascular events[23].

Hypoglycemia was a secondary endpoint of the ADVANCE trial. It was defined as a BG level of < 2.8 mmol/L (< 50.5 mg/dL) or the presence of typical symptoms and signs of hypoglycemia without other apparent causes. Patients with transient dysfunction of the central nervous system requiring external help for treatment were considered to have severe hypoglycemia. During the 5-year follow-up severe hypoglycemia was uncommon. However, it was significantly more common in the intensive-control than standard-control group (2.7% *vs* 1.5%)[23].

Excluded linagliptin trials

Cardiovascular and Renal Microvascular Outcome Study With Linagliptin trial: The other study of linagliptin *vs* placebo that reported both HE and MACE as outcomes was the landmark "Cardiovascular and Renal Microvascular Outcome Study With Linagliptin" (CARMELINA) trial. This study was excluded from the narrative synthesis because it evaluated HE and MACE in 6979 T2D patients with high CV and renal risk[8]. However, given that this was a landmark trial, it is discussed in the excluded linagliptin studies section.

This study evaluated HE and MACE in 6979 T2D patients with high CV and renal risk[8]. The trial, designed as a non-inferiority trial of linagliptin *vs* placebo, assessed the first occurrence of the composite of MACE as a primary endpoint and hypoglycemia was assessed as an AE. The outcomes of interest were well defined according to predefined criteria. After a median follow-up of 2.2 years, MACE occurred in 12.4% and 12.1% in the linagliptin and placebo groups, respectively, and the difference was statistically significant[8]. The frequency of confirmed HEs including severe hypoglycemia in the linagliptin *vs* placebo group was 15.9% *vs* 16.4%. HE in the placebo group was due to rescue medications that were allowed to control hyperglycemia[8].

CAROLINA trial: In the CAROLINA trial, 6042 subjects with T2D and HbA1c of 6.5%-8.5% who were at high CV risk (had established CV disease and renal impairment but not end stage renal disease) were randomized to linagliptin at 5 mg/d (n = 3028) *vs* glimepiride at doses of 1-4 mg/d (n = 3014)[9]. After a mean follow-up of 6.3 years, the primary outcome of the trial (MACE) occurred in 11.8% of subjects in the linagliptin arm *vs* 12% of subjects in the glimepiride arm, and the difference was statistically significant[9]. At least one HE occurred in 10.6% *vs* 37.7% of participants in the linagliptin *vs* glimepiride group, respectively[9].

DISCUSSION

There were no CVOT trials for gliclazide. The landmark ADVANCE trial[23] compared two levels of glycemic control, intensive (HbA1c < 6.5%) *vs* standard (managed with oral GLD according to local practice). It was not a CV safety trial of gliclazide, but the trial did show that the primary endpoint of the composite of microvascular and macrovascular events was significantly reduced by 18.1% in the

intensive control gliclazide arm.

On the other hand, CV safety of linagliptin has been demonstrated by two RCTs, namely the CARMELINA[8] (*vs* placebo) and the CAROLINA[9] (*vs* glimepiride, a SU) trials. These dual randomized CVOT linagliptin trials in T2D patients (CARMELINA[8] and CAROLINA[9]) showed that linagliptin was non-inferior to placebo and glimepiride, respectively, for the composite of MACE.

This CV safety of gliclazide in the ADVANCE trial and of linagliptin in the CARMELINA and CAROLINA trials was demonstrated in patients at high risk of CVD. Hence, gliclazide and linagliptin can be considered as oral GLD that can be given safely in T2D patients with CVD or at high risk of CVD.

In this context, the two RCTs comparing gliclazide with vildagliptin, a DPP4 inhibitor[34,35], were not powered to assess hypoglycemia and MACE as outcomes. Instead, they reported these as AEs. However, neither trial reported a significant difference in CV safety and/or HE incidence between gliclazide and vildagliptin. In this context, it is important to note that linagliptin and vildagliptin belong to two different classes of DPP4 inhibitors[55]. Hence, it is important to compare gliclazide with linagliptin.

Also, all SUs do not have the same CV risks. SUs like glyburide/glibenclamide inhibit an ATP-sensitive potassium channel in the heart and pancreas and are therefore associated with increased CV risk as compared to gliclazide, which selectively inhibits ATP-sensitive potassium channels only in the pancreas[56]. The CARMELINA trial compared linagliptin with glimepiride. However, the double-blind head-to-head comparison GUIDE study showed that compared to glimepiride, gliclazide had a better safety profile and resulted in 50% fewer HEs[2]. The frequency of CV AEs was similar in both glimepiride and gliclazide groups and judged by the investigator as not related to the treatment[2].

Strengths and limitations

Literature was searched using only free resources such as MEDLINE and Google scholar. Hence, the SR is likely to have missed some important articles on the paid sites. The strict inclusion and exclusion criteria is likely to have filtered out important RCTs and real-world studies that could have added value to the CV and hypoglycemia profile of these two drugs. This SR was also limited by its reporting style of narrative synthesis. However, as explained under the "Narrative synthesis of data" section, there were no trials comparing gliclazide and linagliptin. Hence, gliclazide and linagliptin studies were independently assessed for the outcomes of interest. For most studies included in the narrative synthesis, except the CARMELINA[8], ADVANCE[23] and Diamicron MR in NIDDM: Assessing Management and Improving Control 1 study[33], hypoglycemia, MI and other CV events were reported as cause of exclusion from the study or withdrawal from study and non-inclusion in analysis. Hence, these trials looked at outcome of interest in patients, not at risk of CV and renal events.

Filtering of gliclazide trials by the year (2008) resulted in inclusion of trials in the later trajectory of gliclazide compared to linagliptin trials that were in the earlier stage of drug trajectory. This resulted in exclusion of five randomized gliclazide clinical trials that reported the outcomes of interest in the initial drug trajectory[2,57-60] These included trials compared various gliclazide formulations[57,60] and trials comparing gliclazide with other SUs such as the GUIDE Study[2] and with thiazolidinediones (QUARTET Study Group)[58]. However, none of these RCTs included a DPP4 inhibitor as a comparator. Hence, their exclusion did not affect the narrative synthesis.

All the records included in this study were RCTs or a factorial randomized design. Hence, quality of records included was good.

CONCLUSION

Although, the head-to-head comparative clinical data between gliclazide and linagliptin is lacking, both the drugs have shown effective glycemic control along with CV safety in patients with T2D. In resource limited settings, SUs are commonly used as the first add-on therapy after metformin because of cost constraints. In these settings, there is a need to compare modern Sus like gliclazide, which have a cardiac-sparing action, with drugs with established CV safety in CVOT such as DPP4 inhibitors. Future RCTs may confirm the comparative CV outcomes between gliclazide and linagliptin and other DPP4 inhibitors.

ARTICLE HIGHLIGHTS

Research background

Type 2 diabetes (T2D) patients are at increased cardiovascular and treatment-related hypoglycemia risk. Various guidelines recommend dipeptidyl peptidase-4 (DPP4) inhibitors as the first add-on therapy to metformin in T2D due to their confirmed cardiovascular benefits demonstrated through cardiovascular outcome trials. However, in resource limited countries like India, newer sulfonylureas, like gliclazide and glimepiride, are the most commonly used glucose-lowering drugs in T2D due to their low cost.

Gliclazide and glimepiride have similar glycemic efficacy, but gliclazide has a 50% lower hypoglycemia risk.

Research motivation

A landmark cardiovascular outcome trial demonstrated the cardiovascular safety of glimepiride against linagliptin (a DPP4 inhibitor). However, the cardiovascular safety of gliclazide *vs* linagliptin has not been established through cardiovascular outcome trials. If the cardiovascular safety and lower hypoglycemia risk of gliclazide is established *vs* linagliptin, it will help physicians prescribe it with assurance of safety for their patients.

Research objectives

To assess the cardiovascular safety and hypoglycemia risk of gliclazide as compared to linagliptin (and other DPP4 inhibitors). The objective was to assess whether gliclazide was as safe as the guideline recommended DPP4 inhibitor (linagliptin) in providing cardiovascular safety and lowering hypoglycemia risk in T2D. This systematic review was likely to help provide assurance regarding cardiovascular and hypoglycemia safety of gliclazide in T2D as compared to costlier DPP4 inhibitors.

Research methods

This systematic review followed the current Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines to analyze all the clinical studies published from 2008 through the present that compared the cardiovascular safety and hypoglycemia risk of the two drugs in patients with T2D with no cardiovascular disease. Using keywords such as “linagliptin”, “Gliclazide”, “hypoglycemia”, “myocardial infarction”, and “cardiovascular death”, we searched the databases MEDLINE and Google Scholar. Two independent reviewers assessed the trials included using the current Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines for systematic reviews. We included only evidence designated high quality by the Oxford Center for Evidence-based Medicine-Levels of Evidence. The primary outcomes compared were major adverse cardiovascular events and hypoglycemia risk.

Research results

We could not find any trial comparing gliclazide with linagliptin, either as monotherapy or as add-on therapy to metformin. The cardiovascular safety of gliclazide in the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial and of linagliptin in the Cardiovascular and Renal Microvascular Outcome Study With Linagliptin (CARMELINA) and CARdiovascular Outcome study of LINAgliptin *vs* glimepiride in patients with T2D (CAROLINA) trials were excluded from the comparative analysis as these trials demonstrated cardiovascular and hypoglycemia benefits in patients at high risk of cardiovascular disease. However, since these are landmark trials, their results are important and hence described in detail as a separate section. The final analysis included five gliclazide and three linagliptin trials (total eight studies) that individually studied the outcomes of interest in T2D patients with no established cardiovascular disease. Statistical comparisons of the results were not possible as the trials had different designs, different definitions of major adverse cardiovascular events and hypoglycemia and were conducted in different patient populations. Hence, no direct comparisons were possible. The trials were therefore described individually, and their results were compared through narrative synthesis. We assessed that both drugs were effective in achieving the desired glycemic control and had low major adverse cardiovascular events and hypoglycemia risk in adult patients with no cardiovascular disease.

Research conclusions

Gliclazide can be considered as an effective and safe glucose-lowering drug in T2D patients with no established cardiovascular disease but at high risk of cardiovascular disease due to their T2D status.

Research perspectives

Future randomized controlled trials comparing gliclazide with linagliptin or DPP4 inhibitors can add value to the findings of this systematic review.

ACKNOWLEDGEMENTS

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work and have given final approval for the version to be published. The authors thank Dr. Punit Srivastava and Dr. Kokil Mathur of Mediception Science Pvt. Ltd (www.mediception.com) for providing medical writing support in the preparation of this manuscript.

FOOTNOTES

Author contributions: Mohan V, Wangnoo S, Das S, Dhediya R and Gaurav K read and approved the final manuscript; all authors contributed equally.

Conflict-of-interest statement: There are no conflicts of interest to report.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Country/Territory of origin: India

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S-Editor: Chen YL
L-Editor: Filipodia
P-Editor: Chen YL

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