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EDITORIAL

Role of lipid-lowering agents in the management of diabetic retinopathy

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Abstract

Diabetic retinopathy affects a substantial proportion of patients with diabetes mellitus (DM) and is the leading cause of blindness in working-aged adults. Even though the incidence of diabetic retinopathy has declined in the last decades, its prevalence increased and is expected to rise further as a result of the increasing incidence of type 2 DM (T2DM) and the longer life expectancy of patients with DM. The pathogenesis of diabetic retinopathy is multifactorial. Some observational studies suggested an association between dyslipidemia and the development and progression of retinopathy in patients with DM but others did not confirm this association. Regarding lipid-lowering agents, studies that evaluated the role of statins in the management of these patients are mostly small and yielded discrepant results. Large randomized studies with statins in patients with T2DM showed no benefit of these agents on diabetic retinopathy but were not designed to address this effect. In contrast, both preclinical data and two large randomized controlled studies, the FIELD and the ACCORD trial, showed that fenofibrate delays the progression of diabetic retinopathy. Even though the mechanisms underpinning this favorable effect are not entirely clear, these findings suggest that fenofibrate might represent a useful tool for the management of diabetic retinopathy.

Key words: Diabetes mellitus; Lipid-lowering agents; Statins; Fibrates; Ezetimibe; Colesevelam; Retinopathy

Core tip: Even though it is unclear whether dyslipidemia is implicated in the pathogenesis of diabetic retinopathy, both preclinical data and two large randomized controlled studies showed that treatment with fenofibrate delays the progression of diabetic retinopathy. In contrast, statins do not appear to play a role in the management of this complication despite the promising findings of animal studies.

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INTRODUCTION

Diabetic retinopathy affects 27%-40% of patients with diabetes mellitus (DM)[1-4] and is the leading cause of blindness in working-aged adults[5]. Even though the incidence of diabetic retinopathy has declined in the last decades[6,7], its prevalence increased and is expected to rise further as a result of the increasing incidence of type 2 DM (T2DM) and the longer life expectancy of patients with DM[1,8].

The pathogenesis of diabetic retinopathy is complex and involves many different pathways (Figure 1). Hyperglycemia is a major culprit and induces: (1) accumulation of advanced glycation end-products (AGE), which promote retinal pericyte loss; (2) inflammation, which increases vascular permeability and apoptosis of endothelial and neural cells; (3) protein kinase C (PKC) activation, which increases expression of matrix proteins and induces pericyte apoptosis; (4) accumulation of sorbitol through the polyol pathway, which damages endothelial cells and pericytes; (5) activation of the renin-angiotensin system, which induces vascular endothelial growth factor (VEGF) expression; and (6) oxidative stress, which further increases AGE accumulation and activation of PKC and the polyol pathway[9,10]. The above pathogenetic mechanisms result in endothelial dysfunction, which in turn induces retinal ischemia and increases retinal vascular permeability[9,10]. The former up-regulates the expression of VEGF, erythropoietin, carbonic anhydrase and growth hormone, which in turn promote neovascularization[9,10]. On the other hand, increased retinal vascular permeability might result in macular edema[9,10]. In addition to microvascular disease, neuroretinal damage is also implicated in the development and worsening of diabetic retinopathy, since increased neuronal apoptosis is observed in the retina in these patients[9,10]. Accordingly, tight glycemic control and aggressive blood pressure-lowering, particularly with blockers of the renin-angiotensin system, considerably reduce the risk for diabetic retinopathy[11-14]. However, only a minority of patients with DM achieves glycemic and blood pressure targets[15,16]. Moreover, hyperglycemia and hypertension only partly account for the risk of development and progression of diabetic retinopathy, suggesting that other pathogenetic mechanisms also play a role[9,10,17].

LIPIDS AND DIABETIC RETINOPATHY

In this context, some observational studies suggested that elevated serum low-density lipoprotein cholesterol (LDL-C) levels are associated with increased incidence for diabetic retinopathy[18-20]. However, others did not confirm this association[21-23]. Elevated serum triglyceride levels also increased the risk for diabetic retinopathy in some reports[22,24] but not in others[23] whereas elevated high-density lipoprotein cholesterol levels were protective in some studies[22] but not in others[20,23]. In addition, perivascular deposition of lipid-laden macrophages has been reported in the retina of patients with diabetic retinopathy[25]. Moreover, small uncontrolled studies suggest that low-fat diet reduces hard exudates[26,27].

STATINS IN THE MANAGEMENT OF DIABETIC RETINOPATHY

Statins act by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. This results in reduced serum LDL-C levels but also inhibits the mevalonate pathway, which results in reduced production of isoprenoids, including farnesyl pyrophosphate and geranylgeranyl pyrophosphate[28]. The latter leads to reduced prenylation (*i.e.*, addition of farnesyl and geranylgeranyl to cysteine residues of proteins), which modulates a host of pathogenetic mechanisms involved in diabetic retinopathy, including inflammation, oxidative stress, angiogenesis and endothelial dysfunction[28]. In retinal endothelial cells, statins exhibit antiangiogenic actions by suppressing VEGF phosphorylation[29]. In retinal pigment epithelial cells, statins decrease the expression of matrix metalloproteinases (MMP), preventing the breakdown of the blood-retinal barrier[30]. Moreover, in animal models of diabetic retinopathy, treatment with statins prevented the upregulation of VEGF and preserved the blood-retinal barrier by exerting antioxidant[31-33] and anti-inflammatory effects[34,35]. In other preclinical studies, statins induced endothelium-dependent, nitric oxide-mediated vasodilation in retinal arteries[36].

In subjects without DM, statins improve endothelial function in the choroidal vasculature[37] and increase the blood flow in retinal arteries and veins[38]. In patients with diabetic retinopathy, statins reduce vascular resistance in the ophthalmic and central retinal arteries[39]. In addition, vitreous concentrations of VEGF, angiopoietin-2, MMP-9 and transforming growth factor β1 are lower in patients with diabetic retinopathy treated with statins[40].

A recent observational study showed that treatment with statins prior to vitrectomy is associated with greater improvement in visual acuity, particularly in patients who also underwent laser photocoagulation or received treatment with antibodies against VEGF and in those who had macular edema, vitreous hemorrhage, retinal detachment or proliferative retinopathy[41]. Treatment with statins also reduced the risk for repeat vitrectomy[41]. In a recent nationwide matched cohort study from Denmark (n = 62716), patients who were using statins prior to the diagnosis of DM had 40% lower risk for developing diabetic retinopathy[42]. However, other observational studies did not show a protective role of statins against retinopathy in patients with established DM[21,43].

Early case reports in patients with either type 1 DM (T1DM) or T2DM reported reduction of hard exudates and microaneurysms after treatment with statins[25,44]. Studies

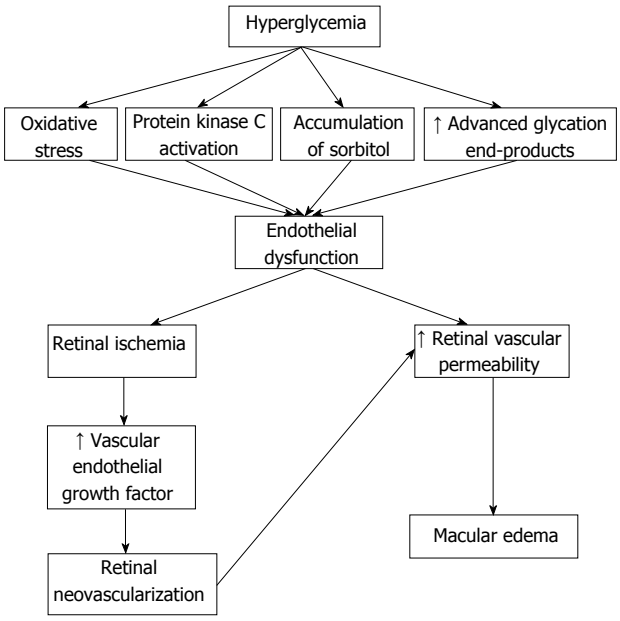

Figure 1 The most important pathogenetic mechanisms involved in the development of diabetic retinopathy.

evaluating the effects of statins on diabetic retinopathy are shown in the Table 1. In a randomized study in 50 patients with T1DM or T2DM, treatment with simvastatin for 6 mo retarded the progression of retinopathy compared with placebo[45]. In an uncontrolled study in 18 patients with diabetic maculopathy, treatment with atorvastatin for 12 mo reduced hard exudates and fluorescein leakage[46]. In a randomized study in 30 patients with T2DM and macular edema, treatment with atorvastatin for 18 wk reduced the number of hard exudates compared with no lipid-lowering treatment but did not affect macular edema or visual acuity[47]. In addition, in a more recent randomized study in 30 patients with macular edema, treatment with atorvastatin for 6 mo had no effect on hard exudates, macular edema or visual acuity compared with no lipid-lowering treatment[48].

In addition to the conflicting results of these observational and small interventional studies, randomized, placebo-controlled trials did not show a beneficial effect of statins on diabetic retinopathy. In the Collaborative Atorvastatin Diabetes Study, treatment with atorvastatin 10 mg/d for 3.9 years had no effect on the risk of laser therapy or progression of retinopathy in 2838 patients with T2DM[49]. In the Heart Protection Study (n = 5963 patients with T2DM followed-up for 4.8 years), the incidence of laser treatment also did not differ between patients treated with simvastatin 40 mg/d and those who received placebo[50]. However, neither of these studies was designed to evaluate the effect of statins on diabetic retinopathy.

FIBRATES IN THE MANAGEMENT OF DIABETIC RETINOPATHY

Fibrates act by inhibiting the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα). PPARα activation not only mediates the lipid-lowering effects of fibrates but also results in inhibition of inflammation by suppression of nuclear factor κB and by direct binding to genes encoding proinflammatory cytokines[51]. Fenofibrate prevents the apoptosis of retinal endothelial cells[52] and of retinal pigment epithelial cells[53]. It also reduces retinal vascular permeability by exerting antiinflammatory effects and by suppressing the upregulation of fibronectin and collagen Ⅳ in the basal membrane of retinal capillaries[54,55]. Moreover, fenofibrate prevents the disruption of the retinal pigment epithelium[56]. Similar with statins, fenofibrate induces endothelium-dependent, nitric oxide-mediated vasodilation in retinal arteries[57].

Early studies reported a decrease in hard exudates after treatment with clofibrate[58]. More importantly, in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial (n = 9795 patients with T2DM), treatment with fenofibrate for 5 years reduced the need for laser photocoagulation by 31% (P = 0.002) and the risk of progression of retinopathy by 79% (P = 0.004) compared with placebo[59]. It was estimated that 17 patients with retinopathy had to be treated with fenofibrate for 5 years to prevent one laser treatment[59]. Interestingly, these benefits were apparent within 8 mo of initiation of fenofibrate treatment, suggesting that other mechanisms than lipid-lowering might be implicated[59]. However, fenofibrate had no effect on the development of retinopathy in patients without retinopathy at baseline and did not prevent the deterioration of visual acuity[59]. More recently, in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) Eye study (n = 2856 patients with T2DM), treatment with fenofibrate for 4 years reduced the rate of progression of retinopathy by 40% (P = 0.006) compared with placebo but again did not affect the occurrence of moderate vision loss[60].

OTHER LIPID-LOWERING AGENTS IN THE MANAGEMENT OF DIABETIC RETINOPATHY

In an early study, administration of omega-3 fatty acids to streptozotocin-induced diabetic rats did not affect pericyte loss and increased the formation of acellular, occluded capillaries in the retina[61]. However, in more recent animal studies, treatment with omega-3 fatty acids preserved retinal function[62,63]. However, there are no studies that evaluated the effects of omega-3 fatty acids on diabetic retinopathy in humans. There are also no studies evaluating the safety and efficacy of colesevelam or ezetimibe in animal models or patients with diabetic retinopathy.

CONCLUSION

It is unclear whether dyslipidemia is implicated in the pathogenesis of diabetic retinopathy. Observational

Table 1 Studies evaluating the effects of statins and fibrates on diabetic retinopathy

Ref.	Type of diabetes mellitus	*n*	Treatment	Dose (mg/d)	Follow-up (mo)	Outcome
[45]	1 and 2	50	Simvastatin	20	6	Delayed progression of retinopathy
[46]	2	18	Atorvastatin	20	12	Reduction of hard exudates and fluorescein leakage
[47]	2	30	Atorvastatin	10	4.5	Reduction of hard exudates but no effect on macular edema or visual acuity
[48]	2	30	Atorvastatin	20	6	No effect on hard exudates, macular edema or visual acuity
[49]	2	2838	Atorvastatin	10	47	No effect on the incidence of laser treatment or progression of retinopathy
[50]	2	5963	Simvastatin	40	58	No effect on the incidence of laser treatment
[59]	2	9795	Fenofibrate	200	60	Reduction of the need for laser photocoagulation and the risk of progression of retinopathy but no effect on the development of retinopathy in patients without retinopathy at baseline or on the risk of deterioration of visual acuity
[60]	2	2856	Fenofibrate	160	48	Reduction of the rate of progression of retinopathy but no effect on the occurrence of moderate vision loss

studies reported conflicting findings regarding the association between lipids and development or progression of diabetic retinopathy. Moreover, studies that evaluated the role of statins in the management of these patients are mostly small and yielded discrepant results. Large randomized studies with statins in patients with T2DM showed no benefit of statins on diabetic retinopathy but were not designed to address this effect. In contrast, both preclinical data and two large randomized controlled studies, the FIELD and the ACCORD trial, showed that fenofibrate delay the progression of diabetic retinopathy. Even though the mechanisms underpinning this benefit are still not entirely clear, these findings suggest that fenofibrate might represent a useful tool for the management of this diabetic retinopathy.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Amount of polyhydramnios attributable to diabetes may be less than previously reported

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Abstract

AIM

To evaluate the frequency and the quantity of polyhydramnios attributable to diabetes in pregnancy.

METHODS

The charts of patients with a four-quadrant amniotic fluid index (AFI) $\geqslant$ 20 cm and either a diagnosis of diabetes or a diabetes screening test during the index pregnancy were retrospectively reviewed. AFI was stratified into 5 categories and the frequency of diabetes was evaluated for each group. The frequency of polyhydramnios attributable to diabetes was compared to the frequency of polyhydramnios in the setting of fetal anomalies or no known cause.

RESULTS

One thousand five hundred and forty-five patients were included in the study. Eight point five percent (n = 131) had diabetes and no other cause for polyhydramnios. Eleven point two percent (173) had antenatally diagnosed anomalies. For all categories of AFI except the largest (> 40.9 cm) the most common cause of polyhydramnios was idiopathic. In patients with diabetes the AFI was most likely to be between 26 cm and 35.9 cm.

CONCLUSION

The rate of polyhydramnios in this study is 8.5%. Patients with diabetes most commonly have mild polyhydramnios between 26 and 35.9 cm of fluid on a four-quadrant AFI.

Key words: Gestational diabetes; Amniotic fluid index; Diabetes in pregnancy; Polyhydramnios

Core tip: A finding of polyhydramnios has been considered an indicator to test the mother for the presence

of diabetes. This is based on reports in the literature of a rate of polyhydramnios due to diabetes between 15% and 25%. This study identified a rate of polyhydramnios associated with diabetes of only 8.5%. This is half of the amount previously reported. Additionally, in patients with diabetes this study found that most had mild polyhydramnios between 26-35.9 cm of fluid on a four-quadrant amniotic fluid index.

Moore LE. Amount of polyhydramnios attributable to diabetes may be less than previously reported. *World J Diabetes* 2017; 8(1): 7-10 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i1/7.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i1.7

INTRODUCTION

Polyhydramnios is defined as a 4-quadrant amniotic fluid index (AFI) > 24 cm or a single maximum vertical pocket > 8 cm. Up to 2% of all pregnancies have an excess amount of fluid meeting the criteria for polyhydramnios[1,2]. It is reported in the literature that approximately sixty percent of polyhydramnios is idiopathic; twenty percent of polyhydramnios can be attributed to poorly controlled or undiagnosed diabetes and the remainder of cases of polyhydramnios are associated with fetal anomalies[2-4]. Several adverse outcomes have been associated with polyhydramnios including preterm labor and rupture of membranes. The primary goal of the study was to evaluate the frequency of polyhydramnios associated with diabetes. Subjectively due to the large of amount of polyhydramnios seen in our clinics and our mainly Hispanic population with a high endemic rate of diabetes, we hypothesized that the frequency of polyhydramnios attributable to diabetes would be greater than the twenty percent quoted in the literature. The secondary goal was to identify a quantity of polyhydramnios associated with diabetes in comparison to other causes. In our review of the literature, no studies have evaluated differences in the quantity of fluid stratified by causation. We hypothesized that the quantity of polyhydramnios associated with diabetes would be different than the quantity of polyhydramnios associated with fetal anomalies or due to an idiopathic process.

MATERIALS AND METHODS

This retrospective cohort study was carried out at the University of New Mexico teaching hospital in Albuquerque, New Mexico, United States of America. This study was reviewed by the Human Research Review Committee (HRRC) at the University of New Mexico and was assigned HRRC#10-418. Patients receiving an ultrasound in the prenatal diagnosis unit from 2009-2012 were included in the study. All patients included in the study were > 28 wk of gestation. The gestational age was chosen so that the majority of patients would have received screening for gestational diabetes which is traditionally performed between 24-28 wk.

Table 1 Demographics

	Data
Hispanic	92% (1426)
Native American	6.7% (105)
Other	1% (14)
Age (yr)	33 ± 5.2
BMI	31 ± 6.1
EGA at diagnosis (wk)	32 ± 2.1

BMI: Body mass index; EGA: Emirates Global Aluminium.

The 4-quadrant AFI for each patient was measured by a registered diagnostic sonographer using the method initially described by Phelan *et al*[5] in which the maternal abdomen is divided into 4 quadrants using the linea nigra as a midline and the umbilicus to define the crossing X-axis. The largest vertical pocket of fluid in each quadrant was measured and the sum of the four measurements was used as the AFI. Associated images of quadrant measurements were reviewed for each patient by a board certified perinatologist.

Patients were included in the study if they received prenatal care at the University of New Mexico, had been tested for diabetes or had a diagnosis of preexisting diabetes, and if on any ultrasound after 28 wk a 4-quadrant AFI was 20 cm or greater. The lower limit of 20 cm of fluid was chosen because this was the cutoff used in the first paper describing the four-quadrant AFI[5]. The frequency of polyhydramnios attributable to diabetes was compared to the frequency associated with fetal anomalies and in patients with no known cause of polyhydramnios.

In the planned secondary analysis, the AFI was stratified into 5 groups; Group A = 20-25.9 cm; Group B = 26-30.9 cm; Group C = 31-35.9 cm; Group D = 36-40.9 cm and Group E > 40.9 cm. The frequency of diabetes was evaluated for each group.

Statistical analysis

Statistical analysis was performed using SAS version 9.2 (SAS institute, Cary, North Carolina). Categorical variables are listed as frequencies and percentages. Continuous variables are presented as mean with standard deviation. The association between categorical variables was analyzed using χ^2 and Fisher's exact test. The frequency of polyhydramnios due to diabetes was assumed to be 20%. We found a frequency of 8.5%. A post hoc power analysis indicated a > 80% power to detect the study hypothesis with a significance rate of 0.05 using a sample size of 1545 patients.

RESULTS

One thousand five hundred and forty-five patients had a 4-quadrant AFI ⩾ 20 cm. Demographics are shown in

Table 2 Results of primary analysis: Quantity of fluid stratified by causation

Amniotic fluid index (*n*)	Diabetic *n* (%)	Anomalies *n* (%)	Idiopathic *n* (%)	*P* value for incidence of diabetes
A: 20-25.9 cm (1261)	7.06 (89)	7.1 (90)	85 (1082)	
B: 26-30.9 cm (199)	15.08 (30)	20.6 (41)	64 (128)	*P* = 0.0002 (B compared to A)
C: 31-35.9 cm (53)	18.87 (10)	41.5 (22)	39 (21)	*P* = 0.002 (C compared to A)
D: 36-40.9 cm (18)	5.56 (1)	38.8 (7)	55 (10)	*P* = 0.9 (D compared to A)
E: > 40.9 cm (14)	7.14 (1)	92.9 (13)	0 (0)	*P* = 0.9 (E compared to A)
				P = 0.85 (E compared to D)

Table 1. The majority of patients in the study (92%) were Hispanic. The mean BMI for study was 31 (range 18-52). 8.5% (*n* = 131) of patients were diabetic. Ninety-four patients had gestational diabetes. Thirty-seven patients had pre-existing diabetes. One hundred and seventy-three (11.2%) patients had antenatally diagnosed anomalies. There were no fetal anomalies in the patients with diabetes. The mean gestational age at diagnosis of polyhydramnios was 32 completed weeks.

The difference between the incidence of diabetes in Group A with an AFI between 20-25.9 cm and Group B with an AFI between 26-30.9 cm was statistically significant (*P* = 0.0002). The difference in incidence of diabetes between Group A and Group C (AFI between 31-35.9 cm) was also statistically significant (*P* = 0.002). The difference between the incidence of diabetes in Groups D and E was not significant in comparison to Group A (*P* = 0.9) or in comparison to each other (*P* = 0.85). There was no difference in AFI between preexisting and gestational diabetics (*P* = 0.6). These results are shown in tabular form in Table 2.

For all categories of AFI except the largest (> 40.9 cm) the cause was most likely to be idiopathic. At mild levels of polyhydramnios between 26-30.9 cm and 31-35.9 cm the frequency of anomalies was higher than the frequency of diabetes, though the frequency of diabetes was statistically different than in the 20-25.9 cm group. Cases of diabetes clustered between 26 cm and 35.9 cm as shown in Table 2. In the 36-40.9 cm grouping, the rate of diabetes began to taper off and was significantly less than the rate of anomalies. In the > 40.9 cm grouping, no cases were idiopathic, 92.9% was due to fetal anomalies and 7% due to diabetes though this actually amounted to a single patient with diabetes in that grouping.

DISCUSSION

In 1970 Queenan *et al*[3] reported on 358 patients with clinically diagnosed polyhydramnios. Thirty-four percent was idiopathic and 24.6% was due to diabetes. This was the initial study to report the association of diabetes with polyhydramnios and is the basis for the recommendation to rule out diabetes when polyhydramnios is discovered. In 1987, Hill *et al*[6] reported on 102 cases of mild to severe polyhydramnios. In 66.7% of those patients no cause was found. Fourteen point seven percent was due to either gestational or preexisting diabetes.

In our study 8.5% of polyhydramnios was associated with diabetes and 11.2% was due to an anomalous fetus and in 74% of our patients no cause was found. The percentage of polyhydramnios attributable to diabetes is lower in our study than previously reported. The lower limit of polyhydramnios in our study was 20 cm of fluid; If the lower limit was defined as 25 cm then the amount of polyhydramnios attributable to diabetes is even lower at 2.7%. This finding was surprising because the population, which is predominantly Mexican-Hispanic, has a high rate of endemic diabetes and we hypothesized that the amount would be higher than previously reported. A possible explanation is improved glycemic control during pregnancy in comparison to the 1970's when Queenan first reported this association. A limitation of our study is that due to its retrospective nature we were unable to evaluate the degree of glycemic control for patients in the study.

Our goal was to evaluate diabetes associated polyhydramnios. Between groups D and E the difference in the frequency of diabetes was not significant, however the difference in the frequency of anomalies between group D compared to group E was significant (*P* = 0.008). This may indicate that anomalies are more common than diabetes at extreme levels of polyhydramnios.

To our knowledge no study has looked at the quantity of polyhydramnios associated with diabetes in comparison to other causes. We found that diabetes is most common in the mild range of polyhydramnios between 26 and 35.9 cm on a four-quadrant AFI.

At the lower end in the 20-25.9 cm group the rate of anomalies and the rate of diabetes was the same. Interestingly, recent definitions of polyhydramnios starting at 25 cm of fluid would eliminate this group which contained 68% (*n* = 89) of all the diabetics in the study.

In conclusion, the majority of cases of polyhydramnios associated with diabetes had a 4-quadrant AFI between 26-35.9 cm. Cases above and below that were outliers. The rate of polyhydramnios attributable to diabetes was 8.5%. This is less than reported in previous studies.

COMMENTS

Background

Queenan first reported the association between diabetes during pregnancy and polyhydramnios in 1971. He found that approximately 1 in 4 cases of polyhydramnios was due to diabetes. This was during a time when women with diabetes were advised to avoid pregnancy because outcomes were universally poor.

Research frontiers

The authors hypothesized that the amount of diabetes associated polyhydramnios would be less than the 25% reported by Queenan. Possibly due to improved glycemic control or other factors. This study addresses whether, in

modern times when women with diabetes have excellent pregnancy outcomes, the incidence of polyhdramnios in diabetes remains the same.

Innovations and breakthroughs

This study shows that the amount of polyhydramnios attributable to diabetes is < 10%. This is significantly lower than previously reported.

Applications

In the setting of polyhydramnios, the most likely cause is idiopathic, followed by the possibility of an anomaly. The frequency of polyhydramnios associated with diabetes was less than the frequency of polyhydramnios associated with an anomaly.

Terminology

Amniotic fluid index (AFI): The sum of the measurements of the largest vertical pocket of amniotic fluid in each of the four quadrants of the pregnant uterus; Polyhydramnios: For this study polyhydramnios was defined as a four-quadrant AFI $\geqslant$ 20 cm.

Peer-review

The manuscript is interesting and well written.

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ORIGINAL ARTICLE

Observational Study

Serum levels of undercarboxylated osteocalcin are related to cardiovascular risk factors in patients with type 2 diabetes mellitus and healthy subjects

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Author contributions: Sanchez-Enriquez S and Zepeda-Moreno A designed the research; Ballesteros-Gonzalez IT, Villafán-Bernal JR and Pascoe-Gonzalez S performed the research; Rivera-Leon EA and Llamas-Covarrubias IM contributed new reagents/analytic tools; Bastidas-Ramirez BE and Rivas-Carrillo JD analyzed the data; Alcala-Zermeno JL and Armendariz-Borunda J wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Universidad de Guadalajara, Centro Universitario de Ciencias de la Salud Institutional Review Board.

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Data sharing statement: Technical appendix, statistical code, and dataset will be available from Sergio Sanchez-Enriquez at Dryad repository, who will provide a permanent, citable and open-access home for the dataset.

Manuscript source: Invited manuscript

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Abstract

AIM

To determine a potential relationship between serum undercarboxylated (ucOC) concentration and cardiovascular risk factors in type 2 diabetes (T2D) patients and healthy subjects (HS).

METHODS

A cross-sectional study was conducted on 140 subjects classified into two groups, 70 with T2D and 70 HS. Medical history and physical examination with anthropometric measurements were obtained from all subjects. Body fat percentage was determined by bioelectrical impendency analysis. Serum ucOC concentration was determined by enzyme immunoassay, while serum levels of insulin and hsCRP were obtained using high sensitivity enzyme-linked immunosorbent assay. Insulin resistance was determined using the homeostasis model assessment-IR. Lipid profile [triglycerides, total cholesterol (TC), high-density lipoproteins (HDL-c), low density lipoproteins (LDL-c), very low-density lipoproteins] was determined by spectrophotometry and standard formulas when applicable.

RESULTS

The T2D patient group showed significantly higher values of waist circumference, waist-to-hip ratio, systolic blood pressure (SBP), diastolic blood pressure (DBP), current smoking, and alcohol use when compared to the HS group ($P < 0.05$). We observed a significantly lower serum ucOC concentration in T2D than in HS (1.5 ± 1.4 *vs* 2.3 ± 1.8, $P < 0.05$). In the whole study population, ucOC concentration was inversely correlated with body mass index (BMI) ($r = -0.236$, $P < 0.05$), fasting plasma glucose ($r = -0.283$, $P < 0.01$) and HDL-c ($r = -0.255$, $P < 0.05$); and positively correlated with LDL-c/HDL-c ratio ($r = 0.306$, $P < 0.05$) and TC/HDL-c ratio ($r = 0.284$, $P < 0.05$). In the T2D group, serum ucOC concentration was inversely correlated with BMI ($r = -0.310$, $P < 0.05$) and body-fat percentage ($r = -0.311$, $P < 0.05$), and positively correlated with DBP ($r = 0.450$, $P < 0.01$). In HS group a positive correlation between serum levels of ucOC and SBP ($r = 0.277$, $P < 0.05$) was observed.

CONCLUSION

Serum ucOC is a potential marker for cardiovascular risk in Mexicans because it is related to adiposity parameters, blood pressure and lipid profile.

Key words: Bone; Osteocalcin; Glucose metabolism; Diabetes; Cardiovascular risk

Core tip: Lower levels of undercarboxylated osteocalcin (OC) are found in diabetic patients as this hormone is involved in various glucorregulatory mechanisms; however evidence regarding its role in cardiovascular disease development is still pending. Here we show the correlation between levels of undercarboxylated OC and markers of cardiovascular risk.

Sanchez-Enriquez S, Ballesteros-Gonzalez IT, Villafán-Bernal JR, Pascoe-Gonzalez S, Rivera-Leon EA, Bastidas-Ramirez BE, Rivas-Carrillo JD, Alcala-Zermeno JL, Armendariz-Borunda J, Llamas-Covarrubias IM, Zepeda-Moreno A. Serum levels of undercarboxylated osteocalcin are related to cardiovascular risk factors in patients with type 2 diabetes mellitus and healthy subjects. *World J Diabetes* 2017; 8(1): 11-17 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i1/11.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i1.11

INTRODUCTION

Osteocalcin (OC) is a non-collagenous peptide composed of 49 aminoacids and produced by osteoblasts. Circulating OC has two variants, carboxylated (cOC) and undercarboxylated (ucOC)[1-3]. While cOC has high affinity for calcium ions contained in hydroxyapatite, is actively involved in osteogenesis[4] and enhances osteoclast maturation[5], ucOC functions as a regulatory hormone in glucose metabolism improving insulin sensitivity and secretion, β-cell mass and glucose tolerance[6,7]. Low serum OC levels and the index ucOC/cOC are associated with increased fasting plasma glucose (FPG) and IR in several cross-sectional and prospective studies[8-12] and with risk for developing type 2 diabetes (T2D)[10,13]. On the other hand, bone metabolism, T2D and cardiovascular diseases (CVD) interact with one another through complex mechanisms[14-21]. For example, bone-related proteins like OC, osteopontin (OPN), osteoprotegerin (OPG) and receptor-activated nuclear factor-k B ligand have been correlated with atherosclerotic arteries, suggesting a potential role of bone molecules in the pathophysiology of vascular disease[22,23]. Furthermore, a previous study showed that OC was inversely correlated with peripheral atherosclerosis markers (intima-media thickness and ankle-brachial pulse-wave velocity) in T2D patients[23]. This data suggests that OC might be involved in the development of CVD in T2D patients. However, direct evidence regarding ucOC concentrations and cardiovascular risk factors (CVRF) in humans is limited. The present study was designed to determine the correlation

between ucOC levels and CVRF in T2D and healthy subjects (HS).

MATERIALS AND METHODS

Study population

We performed a cross-sectional analysis of 140 subjects (70 T2D patients and 70 HS) aged 54.0 ± 5.0 and 51.8 ± 7.4, respectively attending the Program for Detection and Treatment of Congenital and Acquired Metabolic Diseases at a university center (Universidad de Guadalajara) in western Mexico. We obtained the medical history and physical examination from all subjects. Blood for biochemical determinations was drawn after an overnight fasting period. Subjects with hepatic, renal, parathyroid and thyroid dysfunction or taking medications known to influence bone or calcium metabolism, such as vitamin D, bisphosphonates, calcitonin, estrogen, tamoxifen or corticosteroids, were excluded.

Ethical considerations

This study was done according to the Ethical Principles for Medical Research Involving Human Subjects (Declaration of Helsinki) and was approved by the University of Guadalajara Ethics Committee. Informed consent was obtained from all participants before enrollment in the study.

Anthropometric and clinical measurements

Anthropometric measurements were performed in all subjects. Height was measured using a stadiometer with ± 0.001 m accuracy (Seca, Hamburg, Germany). Weight and body fat percentage (BFP) were determined using a fixed frequency (50 kHz) bioimpendance analyzer scale (TBF-300 Tanita, Tokyo, Japan) with an applied current of 0.8 mA. Body mass index (BMI) was calculated by Quetelet index (kg/m^2). Waist circumference (WC) was measured using a flexible measuring tape midway between the lowest rib and the superior iliac crest border in the mid axillary line. Hip circumference (HC) was measured around the buttocks at the greater trochanter level. Waist-to-hip ratio (WHR) was calculated by dividing WC/HC. After 10 min in the seated position, blood pressure was measured 3 times using an electronic aneroid sphygmomanometer (Omron, model HEM-7220 LA; Kyoto, Japan); the last 2 measures were averaged for analysis.

Biochemical analysis

Blood samples were collected after a 12-h overnight fast period. Samples were centrifuged at 4000 rpm for 10 min to obtain serum, which was stored at -70 ℃ for later processing. FPG was quantified by the glucose oxidase method (Biosystems, Barcelona, Spain). Serum triglycerides (TG), total cholesterol (TC), cholesterol bound to high-density lipoproteins (HDL-c) and cholesterol bound to low density lipoproteins (LDL-c) were determined by enzymatic colorimetric procedures (Biosystems, Barcelona, Spain). Cholesterol bound to very low-density lipoproteins (VLDLc) was calculated using Friedewald's equation (TG divided by 5)[24]. Fasting serum insulin (FINS) levels were measured by enzyme-linked immunosorbent assay (ELISA) (GenWay Biotech, Inc. San Diego CA, United States). Serum hsCRP concentration was quantified by high sensitivity ELISA (Abcam, CA, United States). Serum ucOC was determined by enzyme immunoassay (Takara Bio, Inc. Otsu, Japan).

Homeostasis model assessment-IR

The IR was estimated by homeostasis model assessment-IR (HOMA-IR) calculated according to the following formula: HOMA-IR = FINS (mU/L) × FPG (mmol/L)/22.5[25].

Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences v17.0 (SPSS, Inc., Chicago, Illinois). Every analysis was performed by a biomedical statistician. The Kolmogorov-Smirnov one-sample test was performed for assessing the sample cumulative distribution. Quantitative continuous variables were presented as mean ± SD. Normally distributed variables were analyzed using the two independent samples *t*-test. To analyze non-normally distributed data the Mann-Whitney *U* test was performed. Pearson's χ^2 test was used for categorical variable analysis (*i.e.*, gender, alcohol use, and current smoking). Pearson correlation coefficient was used to assess the strength of the association between ucOC concentration levels and CVRF. A P value < 0.05 was considered statistically significant.

RESULTS

Characteristics of subjects

Demographic and clinical characteristics of all subjects are shown in Table 1. Significant differences in WC, WHR, systolic blood pressure (SBP), diastolic blood pressure (DBP), current smoking, and alcohol use were observed between groups. Biochemical parameters according to study groups are shown in Table 2. TC, TG, HDL-c, LDL-c, VLDL-c, LDLc/HDL-c ratio, TC/HDL-c ratio, FPG, HOMA-IR, and ucOC were significantly different between groups (P < 0.05).

Correlation between serum levels of ucOC and CVRF

In the whole study population the serum ucOC concentration was inversely correlated with BMI, FPG and HDL-c, while it was positively correlated with both LDL-c/HDL-c ratio and TC/HDL-c ratio. In the T2D group and inverse correlation between serum levels of ucOC, body fat percentage, and BMI were observed. Conversely, serum levels of ucOC were positively correlated with DBP in this group. In the group of HS, a positive correlation between serum levels of ucOC and systolic pressure was observed (Table 3).

ucOC by DBP and SBP quartiles in T2D

Because of the strong correlation found between DBP and ucOC in T2D patients, participants were classified

Table 1 Demographic and clinical characteristics according to study group

Variable	T2D (n = 70) Mean ± SD	HS(n = 70) Mean ± SD	P value
Age (yr)	54.0 ± 5.0	51.8 ± 7.4	NS
Gender M/F	29/41	30/40	NS
Weight (kg)	75 ± 18.1	72.1 ± 14.8	NS
Height (cm)	159.6 ± 10.1	161.1 ± 7.5	NS
BMI (kg/m^2)	29.3 ± 6.5	27.6 ± 5.2	NS
WC (cm)	99.2 ± 18.1	90.7 ± 12.3	< 0.01
WHR	0.93 ± 0.14	0.87 ± 0.08	< 0.01
Fat percentage	34.5 ± 8.5	33.2 ± 8.7	NS
SBP (mmHg)	143.3 ± 21.5	113.3 ± 11	< 0.001
DBP (mmHg)	86 ± 12.3	74.9 ± 7.5	< 0.001
Current smoking n (%)	24 (38.1)	9 (27.3)	< 0.05
Alcohol use n (%)	29 (46)	2 (3.9)	< 0.001
Physical inactivity n (%)	32 (50.8)	31 (60.8)	NS

Statistical significances were determined using Student's t test (for data normally distributed) or Mann-Whitney test (for data not normally distributed) and χ^2 test (for qualitative variables). T2D: Patients with type 2 diabetes mellitus; HS: Healthy subjects; M/F: Male/female; BMI: Body mass index; WC: Waist circumference; WHR: Waist-to-hip ratio; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; NS: Non statistically significant.

Table 2 Biochemical parameters according to study group

Variable	T2D (n = 70) Mean ± SD	HS (n = 70) Mean ± SD	P value
TC (mg/dL)	220.7 ± 84.7	181 ± 35.5	< 0.01
TG (mg/dL)[1]	140.9 (87.6-200.5)	108.0 (84.0-145.0)	< 0.05
HDLc (mg/dL)	43.1 ± 15.5	68.3 ± 18.0	< 0.001
LDLc (mg/dL)	129.8 ± 37.1	108.9 ± 33.1	< 0.05
VLDLc (mg/dL)	31.1 ± 18.6	25.8 ± 20.0	< 0.05
LDLc/HDLc	3 ± 1.7	2.2 ± 0.1	< 0.05
TC/HDLc	4.7 ± 1.9	3.4 ± 1.5	< 0.01
FPG (mg/dL)	161.9 ± 69.5	88.3 ± 9.0	< 0.001
FINS (mcUI/mL)	15.8 ± 7.0	13.8 ± 11.6	NS
HOMA-IR	6.8 ± 4.1	3 ± 2.6	< 0.001
hs-CRP (ng/L)	3 ± 3.1	2.1 ± 0.8	NS
ucOC (ng/mL)	1.5 ± 1.4	2.3 ± 1.8	< 0.05

[1]Values are expressed as median and interquartilar range. Statistical significances were determined using Student's t test (for data normally distributed) or Mann-Whitney test (for data not normally distributed). TC: Total cholesterol; TG: Triglycerides; HDLc: Cholesterol bound to high-density lipoproteins; LDLc: Cholesterol bound to low-density lipoproteins; VLDLc: Cholesterol bound to very low-density lipoproteins; LDLc/HDLc: LDLc/HDLc ratio; TC/HDLc: TC/HDLc ratio; FPG: Fasting plasma glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment-insulin resistance; hs-CRP: High sensitivity C reactive protein; ucOC: Undercarboxylated osteocalcina; NS: Non statistically significant.

by DBP quartiles, and then ucOC serum levels were compared between quartiles. Also patients were classified according to ucOC quartiles and DBP was analyzed in each ucOC quartile. The ucOC serum levels were higher in T2D patients with DBP in Q4 than in Q1 (P < 0.05, Figure 1A). Additionally, DBP was higher in patients with ucOC in Q4 than those with ucOC in Q1 (P = 0.05, Figure 1B).

Table 3 Correlation between serum levels of undercaboxylated osteocalcin and cardiovascular risk factors

Variable	ucOC concentration (ng/mL) Whole population (n = 140)		T2D (n = 70)		HS (n = 70)	
	r	P value	r	P value	r	P value
BMI (kg/m^2)	-0.236	0.023	-0.310	0.046	-0.166	0.244
Body fat (%)	-0.201	0.054	-0.311	0.048	-0.126	0.379
SBP (mmHg)	-0.083	0.431	0.018	0.908	0.277	0.049
DBP (mmHg)	0.155	0.137	0.450	0.003	0.209	0.141
FPG (mg/dL)	-0.283	0.006	-0.286	0.070	-0.110	0.443
HDL-c (mg/dL)	-0.255	0.036	0.117	0.655	-0.096	0.503
LDL-c/HDL-c	0.306	0.015	-0.102	0.697	0.286	0.054
TC/HDL-c	0.284	0.019	-0.158	0.544	0.221	0.120

Correlations were determined using Pearson correlation coefficients. T2D: Patients with type 2 diabetes mellitus; HS: Healthy subjects; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; HDL-c: Cholesterol bound to high-density lipoprotein; LDL-c/HDL-c: Cholesterol bound to low density lipoprotein/HDL-c ratio; TC/HDL-c: Total cholesterol/HDL-c ratio; ucOC: Undercaboxylated osteocalcin.

DISCUSSION

In this study lower levels of ucOC were found in T2D when compared to HS. Although low levels of total OC and ucOC in T2D patients have been previously reported[11,26-28], there is little evidence describing the relationship between serum levels of ucOC and CVRF.

The association between ucOC concentration and T2D is consistent with *in vitro* and *in vivo* studies that link OC to energetic equilibrium[3,4]. According to previous reports, there is an inverse correlation between serum ucOC levels and; IR, BMI, BFP, FPG and HOMA-IR[7,22,23,29-31].

In our study, multiple associations between ucOC levels and CVRF in T2D patients were found. In T2D patients, ucOC concentration was inversely correlated with BMI and BFP and a positively correlated with DBP. In HS, a positive correlation between ucOc and SBP was also established. Additionally, those T2D patients with highest ucOC levels (Q4) had higher DBP than those with the lowest ucOC (Q1).

The inverse relationship between ucOC and BMI was previously reported by Chen *et al*[32] and Tan *et al*[33] in Chinese men, which might be explained by the role of OC in energy metabolism and by the observation that the OC knockout mice model has abnormal levels of visceral fat[6].

This might be the first report indicating a positive relationship between ucOC levels and DBP in T2D patients and a positive correlation of SBP with ucOC in HS. In non-diabetic young adults, Polgreen *et al*[34] found lower SBP in those having cOC and total OC in Q4 than in Q1, but did not report anything regarding ucOC and DBP. Although Chen, Tan and colleagues found a weak inverse correlation between DBP and total OC they did not did not determined ucOC levels[32,33] which

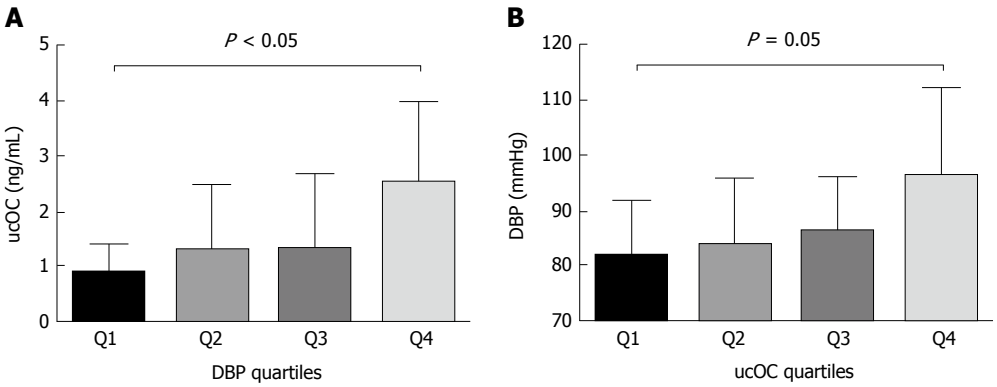

Figure 1 Correlation between undercaboxylated osteocalcin and diastolic blood pressure by quartiles. A: ucOC serum levels by DBP quartiles in type 2 diabetes; B: DBP (mmHg) by quartiles of serum ucOC. ucOC: Undercaboxylated osteocalcin; DBP: Diastolic blood pressure.

would've been desirable since various biological effects outside-the-bone have been endorsed to ucOC[6].

We also observed an inverse association between ucOC levels, BMI, FPG and HDLc, as well as a positive correlation with LDLc/HDLc and TC/HDLc ratios in the whole study population. These data suggest that serum levels of ucOC could be related to CVRF in both HS and T2D patients. These findings agree with the higher ucOC serum levels found by Okura *et al*[35] in hypertensive patients with carotid calcification than in those without carotid calcification, suggesting that ucOC might be a potential biomarker for carotid artery calcification. Our work also agrees with the results published by Kanazawa *et al*[23] who found that total serum OC concentration was negatively associated with atherosclerotic parameters (intima-media thickness and ankle-brachial pulse-wave velocity) independent of other CVRF in diabetic men. Other studies had reported that bone proteins such as matrix Gla protein, OPN and OPG are markers of vascular calcification and are expressed in arteries presenting atherosclerosis[22,23]. Moreover, T2D patients are particularly prone to develop CVD due to the role that diabetes plays in endothelial dysfunction, atherogenesis and vascular calcification[36-39].

Recent studies on animals suggest that OC may have beneficial effects on serum TG levels, but the clinical relevance of this remains elusive[29,31]. Although a significant correlation between serum OC concentration and TG was not found in our study, ucOC levels were inversely correlated with BMI and BFP in the T2D group and with BMI and HDL-c in whole population. This suggests a possible role of ucOC in lipid metabolism regulation. The discrepancy between our study and those conducted in animals could reside in the differences between human and animal lipid metabolism. Numerous studies regarding this issue suggest that TG are positively correlated with bone density while HDL-c is negatively correlated. These observations imply that a common mechanism of lipid and bone metabolism exists. However, more studies need to be performed in order to achieve proper understanding of the pathophysiological relationship between the OC levels and cardiovascular disease[40-49].

Virmani *et al*[41] have defined atherosclerosis as a chronic inflammatory process that can be accelerated by high blood pressure secondary to vasoactive peptides such as angiotensin and endothelin-1. Proinflammatory and prothrombotic risk markers play a very important role in the atheroma formation process, which contributes to the progression of vascular disease in T2D patients by activating inflammatory signaling and oxidative stress, both triggers of the endothelium injury process. Schurgers *et al*[39], found that matrix Gla protein was not carboxylated in atherosclerotic arteries, which could explain the positive correlation between uOC levels and SBP, DBP, LDL-c/HDL-c and TC/HDL-c, well known markers of cardiovascular disease[46,47].

To our knowledge, this is the first study proposing a link between ucOC serum levels and CVRF in a Mexican population. The main limitation of our study is that it cannot identify causal relationship due to its design. Additional studies are needed to determine specifically whether serum ucOC concentration could be considered an independent CVRF. Our findings will generate new hypotheses regarding the role of this protein, not only as a hormone in energy and bone metabolism and its well-studied role in regulation of glucose metabolism, insulin secretion and sensitivity; but also in endothelial dysfunction and as a marker of cardiovascular risk in patients with T2D.

Most reports that studied the relationship between OC and glucose metabolism include only total OC levels determination and data dealing with ucOC in T2D patients in Mexicans is scarce. Our study investigated serum ucOC levels in this population and thus provides information that helps further explore the role of OC in glucose metabolism and CVRF related to T2D.

Serum ucOC levels are related to CVRF in both T2D and HS. Specifically, ucOC is related to adiposity markers and blood pressure, as well as lipid profile. Thus, serum ucOC might be a cardiovascular risk marker in the Mexican population.

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COMMENTS

Background

Undercarboxylated (ucOC) is a non-collagenous peptide involved in various biological processes, including glucose metabolism. The relationship between low levels of ucOC and type 2 diabetes (T2D) is well established and some have proposed ucOC as a marker for metabolic risk. However, the role of ucOC and cardiovascular diseases (a common comorbidity in T2D) has not been well defined.

Research frontiers

Osteocalcin (OC) research now involves fields in energy metabolism, male fertility and brain development. Also a potential receptor to mediate its functions has been proposed, the GPRC6A receptor. However, in cardiovascular disease investigation, there is continuous interest in analyzing the role of OC in atherosclerosis indexes and vascular calcification.

Innovations and breakthroughs

The authors have described various correlations between ucOC levels and markers of cardiovascular risk such as blood pressure, high-density lipoproteins, body mass index and body fat percentage in a Mexican population. Other reports have analyzed the role of ucOC role in arterial calcification in hypertensive patients such as Okura *et al*, however no other group has studied its relationship with cardiovascular risk factors (CVRF) in T2D Mexican patients like in the present report.

Applications

The analysis postulates the possible emergence of ucOC as an independent CVRF in T2D patients.

Terminology

Osteocalcin: Non-collagenous protein that is found in the bone extracellular matrix and in the serum of circulating blood, it is produced by osteoblasts especially in the presence of vitamin D. This hormone exists in two forms: Carboxylated and undercarboxylated; Carboxylated osteocalcin: A form of osteocalcin in which its 3 residues of glutamic acid that reside at the 17, 21 and 24th positions are gamma carboxylated through a vitamin K depend process; Undercarboxylated osteocalcin: Osteocalcin that has less than 3 residues of carboxyglutamic acid.

Peer-review

This is a useful manuscript.

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ORIGINAL ARTICLE

Observational Study

Clinical and dietary predictors of common carotid artery intima media thickness in a population with type 1 and type 2 diabetes: A cross-sectional study

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Abstract

AIM

To determine the clinical and dietary predictors of common carotid artery intima media thickness (CCA IMT) in a cohort of subjects with type 1 and type 2 diabetes.

METHODS

Participants with type 1 (n = 23) and type 2 diabetes (n = 127) had mean and mean maximum CCA IMT measured using B mode ultrasound. Dietary intake was measured using a food frequency questionnaire. Clinical and dietary predictors of mean and mean maximum CCA IMT were determined using linear regression analysis adjusted for potential confounders.

RESULTS

The main predictors of mean and mean maximum CCA IMT were age and weight. After multivariate adjustment there were no dietary predictors of CCA IMT. However,

in subjects that were not prescribed a lipid lowering medication alcohol consumption was positively associated with CCA IMT after multivariate adjustment. No difference existed in CCA IMT between subjects with type 1 or type 2 diabetes once age was adjusted for.

CONCLUSION

CCA IMT was predominantly predicted by age and weight in these subjects with diabetes. The finding that CCA IMT was not different between people with type 1 and type 2 diabetes warrants further investigation in a larger cohort.

Key words: Diabetes; Carotid intima media thickness; Arterial structure; Diet; Lipidomics; Carotenoids

Core tip: This paper examines clinical, dietary and biochemical predictors of common carotid artery intima media thickness (CCA IMT) in a population of participants with type 1 and type 2 diabetes. The only predictors of CCA IMT in this group were age and body weight. After age adjustment CCA IMT was not different in subjects with type 1 or type 2 diabetes.

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INTRODUCTION

In 2013, 8.3% of the world's population had type 1 or type 2 diabetes and the incidence is projected to increase by 55% to 8.8% by 2035[1]. Individuals with type 1 and type 2 diabetes have two to three times the risk of developing cardiovascular disease (CVD) compared to the general population[2-4]. Dietary intake is a modifiable risk factor for CVD with epidemiological studies showing that better diet quality is associated with a reduced risk of CVD in people with diabetes[5,6].

Carotid intima media thickness (IMT) measured using B mode ultrasound, is an early marker of atherosclerosis[7] and predictor of CVD[8]. Type 1 and type 2 diabetes is associated with greater carotid IMT when compared to non-diabetic subjects[9,10]. In individuals with type 1 or type 2 diabetes, per 0.1 mm increase in common carotid artery intima media thickness (CCA IMT), the hazard ratio for a cardiovascular event is 1.12 (95%CI: 1.07-1.16)[11].

Lifestyle factors play a role in the aetiology of carotid IMT progression; an improvement in dietary (saturated fat, fibre, potassium, calcium intake) and lifestyle (smoking and physical activity) factors was associated with lower CCA IMT after 20 years, in a cohort of young adults, independent of demographic factors, medication use and baseline dietary and lifestyle factors[12]. In addition, epidemiological studies show that a higher intake of fruit, olive oil, wholegrains and soluble fibre and lower consumption of saturated fat in favour of polyunsaturated fat is associated with lower carotid IMT[13]. The aim of this study is to determine the clinical and dietary predictors of CCA IMT in a population of adults with type 1 and type 2 diabetes.

MATERIALS AND METHODS

Study methods

This is a cross-sectional study investigating the predictors of CCA IMT in subjects with type 1 and type 2 diabetes. One hundred and fifty subjects were recruited by public advertisement between August 2012 and December 2013. Subjects eligible for inclusion were adults (age > 18 years) with diagnosed type 1 or type 2 diabetes for any duration, managed with diet, oral hypoglycaemic agents and/or insulin. Subjects were excluded if they reported having cancer, unstable CVD requiring intervention, heart failure, significant renal impairment (eGFR < 30 mL/min) or liver disease. Ethics approval was obtained from the University of South Australia Human Research Ethics Committee and all participants provided written informed consent. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12612001052820).

Subjects attended the clinic after an overnight fast on one occasion. Anthropometric measurements and blood pressure were taken by one operator. A blood sample was taken and a random spot urine sample was provided. Ultrasound was used to measure CCA IMT. Participants completed the online version of the Dietary Questionnaire for Epidemiological Studies Version 2 Food Frequency Questionnaire (DQES v2 FFQ) to determine habitual dietary intake.

Anthropometric measurements

Height was measured using a stadiometer (SECA, Hamburg, Germany) to the nearest 0.1 cm while barefoot/flat footwear. Weight was measured to the nearest 0.05 kg using calibrated electronic scales (SECA, Hamburg, Germany) while the participants were barefoot/light footwear and wearing light clothing.

Blood pressure

Clinic brachial blood pressure was measured using an automated sphygmomanometer (SureSigns VS3; Philips, North Ryde, Australia) once the participant had been seated for 5 min. A normal sleeve (16 cm × 52 cm) was used for an arm circumference of 24-32 cm and a large sleeve (16 cm × 70 cm) for an arm circumference of 32-42 cm. A minimum of four consecutive readings were taken at 1 min intervals. The first reading was discarded and the following three consistent measurements, *i.e.*, systolic blood pressure within a range of 10 mmHg, were used.

Common carotid artery intima media thickness

Measurements of the carotid artery were taken using B mode ultra-sound by one operator, with an intra-observer coefficient of variation (CV) of 4.4% (n = 34). Participants were supine with their head positioned at 45 degrees away from the side of the neck being measured. A high resolution ultrasound machine with a 12 MHz transducer was used (Samsung Medison MySono U6, South Korea). A 1 cm region of the IMT on the far wall of the common carotid artery was measured using automatic edge detection software (Samsung Medison MySono U6 Auto IMT, South Korea) as recommended in the Mannheim Carotid Intima-Media Thickness and Plaque Consensus Paper (2004-2006-2011)[14]. Three clips (3 s each) were captured and the mean of 10 measurements taken from each of these clips was averaged for a mean and mean maximum CCA IMT value.

Laboratory measurements

A fasting blood sample was taken and serum total cholesterol, HDL cholesterol, triglycerides, C reactive protein and glucose were measured using a Konelab 20XTi automatic analyser (Thermo Electron Corporation, Louisville, CO, United States) with reagents from Thermo Fisher Scientific (Melbourne, Australia). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula [total cholesterol - high-density lipoprotein (HDL) cholesterol - (triglycerides × 0.45)][15]. Three subjects had a triglyceride level > 4.5 mmol/L which precluded LDL measurement. Serum carotenoids were measured by high performance liquid chromatography according to a previously published protocol[16]. Lipid analysis was performed by liquid chromatography, electrospray ionization-tandem mass spectrometry as previously published[17]. Briefly, 333 individual lipid species from 25 classes were measured. The median intra assay CV was 8%.

A random spot urine sample was provided by participants when they attended the clinic. Aliquots of the urine were taken and frozen at -20 ℃ until analysis of sodium, potassium, creatinine and albumin was done by SA Pathology (Frome Rd, Adelaide, Australia), an accredited commercial laboratory. The albumin to creatinine ratio was calculated from one spot urine sample to determine the presence of micro-albuminuria. Micro-albuminuria was defined as an albumin to creatinine ratio > 2.5 for men and > 3.5 for women[18].

Haemoglobin A1c

The participants were asked to provide the pathology report from their most recent haemoglobin A1c (HbA1c) measurement or the result was sourced from their general practitioner or the pathology company.

Dietary analysis

Habitual dietary intake was measured using the electronic version of the DQES v2 FFQ as previously described[19]. This FFQ has been validated in a population with type 1 and type 2 diabetes[20].

Statistical analysis

Data are presented as mean ± standard deviation (SD) or median (interquartile range) depending on the distribution. Data were checked for normality using Shapiro-Wilk and Kolmogorov-Smirnov values. Independent samples t tests were used to determine differences between subjects with type 1 and type 2 diabetes for continuous variables and χ^2 tests were used for categorical variables. Pearson's correlation was used to determine clinical and dietary predictors of mean and mean maximum CCA IMT. Clinical variables that were correlated (P < 0.1) with CCA IMT were entered into stepwise linear regression (includes both forwards and backwards selection) to determine predictors. For inclusion in the model P < 0.05, bivariate correlations adjusted for age, sex and weight were used to determine the dietary predictors of CCA IMT. Serum carotenoids and serum lipid concentrations were normalised to their respective interquartile ranges to account for the variation in relative abundance in serum. P values for the serum lipid species were corrected for multiple comparisons using the Benjamini Hochberg approach[21]. Analysis was performed using SPSS (version 19, 2010, SPSS Inc, Chicago, IL). Statistical significance was set at P < 0.05.

RESULTS

Subjects were 150 free-living people with diagnosed type 1 or type 2 diabetes (Table 1). After adjustment for age and weight there was no statistically significant difference between the participants with type 1 or type 2 diabetes with regards to blood pressure, total cholesterol, LDL cholesterol, HbA1c or CCA IMT. The subjects with type 2 diabetes did have lower HDL cholesterol (1.1 ± 0.3 mmol/L *vs* 1.5 ± 0.3 mmol/L; P < 0.001) and higher triglycerides (1.4 ± 1.2 *vs* 0.6 ± 0.4; P < 0.05) when compared to the type 1 subjects. The type 1 subjects had been diagnosed with diabetes for a longer duration compared to the participants with type 2 diabetes (19 ± 14 years *vs* 8 ± 7 years; P = 0.001). Fifty-seven percent of the cohort was men.

Age was the strongest predictor of mean CCA IMT (β = 0.006; t = 10.8; P < 0.001) and mean maximum CCA IMT (β = 0.006; t = 10.5; P < 0.001). Figure 1 shows the correlation between age and mean CCA IMT by diabetes type. After age adjustment there was no statistically significant difference between mean (type 1: 0.69 mm; type 2: 0.71 mm; P > 0.05) or mean maximum CCA IMT (type 1: 0.78 mm; type 2: 0.79 mm; P > 0.05) by diabetes type.

In univariate analysis CCA IMT was strongly correlated with age and weakly correlated with smoking pack years, body mass index (BMI), systolic blood pressure, HDL cholesterol and prescription of anti-hypertension and lipid lowering medication (Table 2). After adjustment for age, weight was the only other correlate of mean (β = 0.001; t = 2.4; P = 0.017) and mean maximum CCA IMT (β = 0.001; t = 2.3; P = 0.02). The correlation between

Table 1 Cohort characteristics

Characteristic	Whole cohort (n = 150)	Type 1 diabetes (n = 23)	Type 2 diabetes (n = 127)	P value[1]
Age (yr)	56 ± 14	36 ± 14	60 ± 11	0.0001
Weight (kg)	96.9 ± 21.4	85.6 ± 25.2	98.9 ± 20.0	0.006
Height (m)	1.7 ± 0.1	1.71 ± 0.1	1.71 ± 0.1	0.90
BMI (kg/m^2)	33.1 ± 7.0	29.4 ± 9.3	33.8 ± 6.3	0.005
Sex, male n (%)	86 (57)	9 (39)	77 (61)	0.06
Diagnosed with diabetes (yr)	9.7 ± 9.3	19.1 ± 14.2	8.1 ± 6.9	0.001
Smoking status n (%)				0.25
Never smoked	74 (49)	15 (65)	59 (47)	
Past smoker	68 (46)	7 (31)	61 (48)	
Current smoker	8 (5)	1 (4)	7 (5)	
Smoking pack years[2] (yr)	9.6 ± 15.6	1.7 ± 4.4	11.0 ± 16.5	0.008
Systolic blood pressure (mmHg)	127 ± 14	124 ± 14	128 ± 14	0.15
Diastolic blood pressure (mmHg)	72 ± 10	69 ± 9	72 ± 10	0.18
Mean arterial pressure (mmHg)	90 ± 10	87 ± 8	91 ± 10	0.10
Heart rate (bpm)	74 ± 13	77 ± 10	73 ± 13	0.21
Pulse pressure (mmHg)	56 ± 13	54 ± 15	56 ± 13	0.60
Mean CCA IMT (mm)	0.71 ± 0.13	0.58 ± 0.12	0.73 ± 0.11	< 0.001
Mean maximum CCA IMT (mm)	0.79 ± 0.14	0.66 ± 0.13	0.81 ± 0.12	< 0.001
Prescribed anti-hypertensive medication n (%)	85 (57)	7 (30)	78 (61)	0.006
Prescribed lipid lowering medication n (%)	82 (55)	4 (17)	78 (61)	< 0.001
Diabetes treatment n (%)				< 0.001
None	30 (20.0)	0 (0)	30 (24)	
OHA	65 (43.3)	0 (0)	65 (51)	
Insulin	26 (17.3)	23 (100)	3 (2)	
OHA + insulin	29 (19.3)	0 (0)	29 (23)	
Total cholesterol (mmol/L)	4.1 ± 1.0	4.1 ± 1.0	4.1 ± 1.0	0.99
HDL cholesterol (mmol/L)	1.2 ± 0.4	1.5 ± 0.3	1.1 ± 0.3	< 0.001
LDL cholesterol (mmol/L)	2.4 ± 0.9	2.4 ± 0.9	2.4 ± 0.9	0.97
Triglycerides (mmol/L)	1.3 ± 1.2	0.6 ± 0.4	1.4 ± 1.2	0.003
Glucose (mmol/L)	8.1 ± 3.0	10.1 ± 4.0	7.7 ± 2.6	< 0.001
HbA1c (%) (mmol/mol)	7.3 ± 1.4 (57 ± 16)	7.6 ± 1.1 (60 ± 12)	7.3 ± 1.5 (56 ± 16)	0.27
CRP (mg/L)	2.5 ± 2.2	1.6 ± 1.9	2.6 ± 2.3	0.15
Presence of Microalbuminuria n (%)[3]				
Yes	23 (15)	3 (13)	20 (16)	0.74
No	127 (85)	20 (87)	107 (84)	
Spot urine Na:K	1.3 ± 0.9	1.4 ± 0.9	1.3 ± 0.9	0.62

[1]Type 1 diabetes *vs* type 2 diabetes; [2]Number packs (25 cigarettes)/day × number years smoked; [3]Albumin:creatinine > 2.5 for men and > 3.5 for women[18]. OHA: Oral hypoglycaemic agents; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C reactive protein; HbA1c: Haemoglobin A1c; CCA IMT: Common carotid artery intima media thickness.

weight and CCA IMT was independent of sex. The summarised r^2 values for the mean and mean maximum CCA IMT models (including age and weight) were 0.46 and 0.44, respectively.

When participants prescribed an anti-hypertensive medication were excluded from the analysis, age and weight remained independent predictors of mean and mean maximum CCA IMT and the summarised r^2 values were 0.55 and 0.53, respectively (n = 65). When participants that were prescribed a lipid lowering medication were removed from the analysis, univariate analysis (n = 68) showed that age (r = 0.70; P = 0.001), diabetes type (r = 0.44; P = 0.001), smoking pack years (r = 0.25; P = 0.04), systolic blood pressure (r = 0.43; P = 0.001), pulse pressure (r = 0.33; P = 0.007) and prescription of anti-hypertensive medication (r = -0.39; P = 0.001) were correlated with mean CCA IMT. In a stepwise model age (β = 0.005; t = 6.8; P = 0.001) and systolic blood pressure (β = 0.002; t = 2.4; P = 0.02) were the only predictors of mean CCA IMT. The summarised r^2 value for the model was 0.51.

Median macronutrient intake for the cohort was 21%, 37%, 14% and 40%, respectively for protein, total fat, saturated fat and carbohydrate (Table 3). Dietary intake by food group is presented in Table 4. After adjustment for age, sex and weight there were no dietary predictors of mean or mean maximum CCA IMT, see Tables 3 and 4. When weight was removed from the model there were still no dietary predictors.

Subgroup analysis showed that in those subjects that were not prescribed a lipid lowering medication (n = 68) alcohol consumption was associated with mean (β = 0002; t = 2.5; P = 0.02) and mean maximum (β = 0.002; t = 2.4; P = 0.02) CCA IMT after multivariate adjustment for predictors (age, sex and systolic blood pressure). There were no other dietary predictors of CCA IMT identified in the subjects not prescribed a lipid lowering medication. Subgroup analysis including only subjects that were not prescribed an anti-hypertensive medication (n = 65) showed no dietary predictors of CCA IMT after multivariate adjustment.

The spot urine sodium to potassium ratio was not

Table 2 Predictors of mean and mean maximum common carotid artery intima media thickness in univariate analysis and stepwise linear regression

Predictor	Mean CCA IMT	Mean maximum CCA IMT	Mean CCA IMT			Mean maximum CCA IMT		
	r	*r*	β	*t*	*P*	β	*t*	*P*
Age	0.66[1]	0.65[1]	0.006	10.8	0.0001	0.006	10.5	0.001
Sex	-0.13	-0.14[3]	-			-		
Type of diabetes	0.43[1]	0.41[1]	-			-		
Years since diabetes diagnosis	0.08	0.09	-			-		
Smoking pack years	0.19[2]	0.19[2]	-			-		
Weight	0.16[3]	0.16[3]	0.001	2.4	0.017	0.001	2.3	0.02
BMI	0.18[2]	0.18[2]	-			-		
Systolic blood pressure	0.26[1]	0.27[1]	-			-		
Diastolic blood pressure	-0.01	-0.006	-			-		
Heart rate	-0.24[1]	-0.23[1]	-			-		
Pulse pressure	0.29[1]	0.29[1]	-			-		
Anti-hypertension medication prescription	-0.34[1]	-0.32[1]	-			-		
Lipid lowering medication prescription	-0.30[1]	-0.30[1]	-			-		
Total cholesterol	-0.001	-0.001	-			-		
LDL cholesterol	-0.007	-0.02	-			-		
HDL cholesterol	-0.18[2]	-0.17[2]	-			-		
Triglycerides	0.13	0.14[3]	-			-		
Fasting glucose	0.03	0.03	-			-		
HbA1c	-0.04	-0.04	-			-		
Microalbuminuria	0.15[3]	0.15[3]	-			-		
Summarised r^2 model			0.46			0.44		

[1]$P < 0.01$; [2]$P < 0.05$; [3]$P < 0.1$. HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C reactive protein; HbA1c: Haemoglobin A1c; CCA IMT: Common carotid artery intima media thickness; BMI: Body mass index.

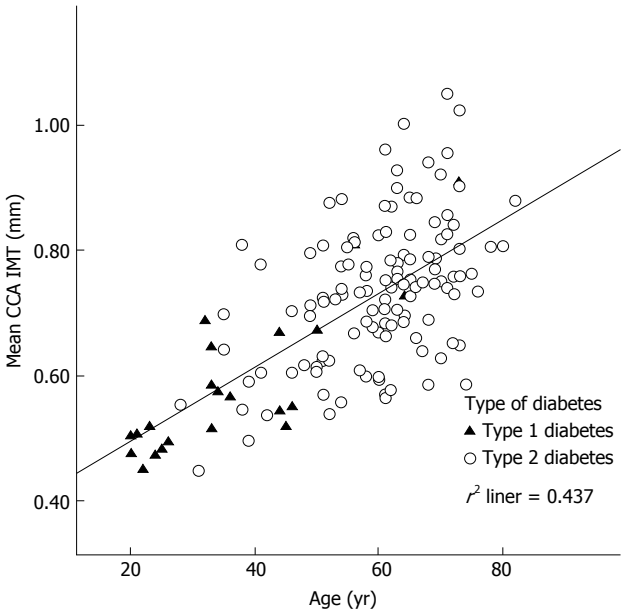

Figure 1 The correlation between age and mean common carotid artery intima media thickness by diabetes type. CCA IMT: Common carotid artery intima media thickness.

associated with mean or mean maximum CCA IMT. No association existed between mean or mean maximum CCA IMT and total serum carotenoids, β-cryptoxanthin, lutein, zeaxanthin, lycopene, α-carotene or β-carotene. There was no correlation between lipid species, expressed as concentration normalised to the interquartile range and mean or mean maximum CCA IMT, after adjustment for predictors and multiple comparisons. Subgroup analysis by anti-hypertensive and lipid lowering prescription showed no differential effect for the association between CCA IMT and serum carotenoids, lipid species and the spot urine sodium to potassium ratio.

DISCUSSION

In this cohort of well-controlled subjects with type 1 and type 2 diabetes the main predictors of mean CCA IMT were age and weight explaining 46% of the variance in the model. After adjustment for age, sex and weight there were no dietary predictors of mean or mean maximum CCA IMT. However, in subjects that were not prescribed a lipid lowering medication alcohol consumption was positively associated with CCA IMT after multivariate adjustment. There was no correlation between serum lipid species or carotenoids and CCA IMT. It was found that after adjustment for age CCA IMT was not different between people with type 1 and type 2 diabetes.

In this population, age was the strongest predictor of CCA IMT. Age is well established as a predictor of CCA IMT in subjects with type 1[10,22] and type 2 diabetes[23]. In addition, weight was a positive predictor of CCA IMT in this study, independent of sex, and has been previously associated with CCA IMT in cohorts with and without diabetes[24,25]. Univariate analysis showed male sex was non-significantly associated with greater mean ($P = 0.12$) and mean maximum ($P = 0.089$) CCA IMT. Previously it has been shown that male sex is associated with greater CCA IMT in people with diabetes[23,24]. Univariate analysis showed that smoking pack years was weakly positively associated with mean and mean maximum CCA IMT but this correlation did not persist after adjustment for age and weight. This is likely to be because less than half of

Table 3 Nutrient intake as reported in the food frequency questionnaire and the correlation with common carotid artery intima media thickness

Nutrient intake	Median (IQR)	Mean CCA IMT		Mean maximum CCA IMT	
		Univariate	Adjusted[3]	Univariate	Adjusted[3]
		r	*r*	*r*	*r*
Energy (kcal)	1652 (1343, 2165)	-0.16[2]	-0.04[2]	-0.15[2]	-0.05[2]
Protein (g/d)	88 (66,112)	-0.18[1]	-0.02[2]	-0.17[1]	-0.02[2]
% E protein	21 (18, 23)	0.02[2]	0.10[2]	0.03[2]	0.11[2]
Total fat (g/d)	66 (52, 92)	-0.18[1]	-0.07[2]	-0.18[1]	-0.08[2]
% E total fat	37 (33, 40)	-0.14[2]	-0.13[2]	-0.16[2]	-0.15[2]
Saturated fat (g/d)	25 (19, 35)	-0.16[1]	-0.07[2]	-0.17[1]	-0.09[2]
% E saturated fat	14 (12, 16)	-0.15[2]	-0.14[2]	-0.16[1]	-0.16[2]
Monounsaturated fat (g/d)	24 (18, 33)	-0.18[1]	-0.07[2]	-0.18[1]	-0.08[2]
% E monounsaturated fat	13 (12, 15)	0.19[2]	-0.09[2]	-0.13[2]	-0.11[2]
Polyunsaturated fat (g/d)	11 (8, 14)	-0.14[2]	-0.06[2]	-0.13[2]	-0.05[2]
% E polyunsaturated fat	6 (5, 7)	0.03[2]	-0.02[2]	0.03[2]	-0.02[2]
Carbohydrate (g/d)	160 (134, 216)	-0.13[2]	-0.06[2]	-0.13[2]	-0.06[2]
% E carbohydrate	40 (36, 44)	0.04[2]	-0.06[2]	0.04[2]	-0.06[2]
Sugar (g/d)	74 (56, 94)	-0.01[2]	0.03[2]	-0.01[2]	-0.03[2]
Fibre (g/d)	20 (16, 26)	-0.12[2]	-0.04[2]	-0.12[2]	-0.05[2]
Sodium (mg/d)	2177 (1696, 2957)	-0.16[2]	-0.06[2]	-0.15[2]	-0.06[2]
Potassium (mg/d)	2771 (2128, 3403)	-0.07[2]	0.03[2]	-0.06[2]	0.04[2]
Alcohol (g/d)	2 (0, 8)	0.06[2]	0.11[2]	0.07[2]	0.11[2]

[1]$P < 0.05$; [2]$P > 0.05$; [3]Adjusted for age, sex and weight. CCA IMT: Common carotid artery intima media thickness.

Table 4 Dietary intake by food group as reported in the food frequency questionnaire and the correlations with common carotid artery intima media thickness

Dietary intake	g/d median (IQR)	Mean CCA IMT		Mean maximum CCA IMT	
		Univariate	Adjusted[4]	Univariate	Adjusted[4]
		r	*r*	*r*	*r*
Total breads and cereals	204 (141, 320)	-0.20[2]	-0.07[3]	-0.18[2]	-0.05[3]
Breakfast cereal	18 (3, 45)	-0.18[2]	-0.18[3]	-0.19[2]	-0.20[3]
Bread	60 (30, 90)	0.06[3]	-0.08[3]	0.06[3]	-0.07[3]
Pasta/rice	59 (32, 109)	-0.28[1]	0.01[3]	-0.27[1]	0.01[3]
Total vegetables/legumes	151 (112, 217)	0.06[3]	0.10[3]	0.06[3]	0.09[3]
Vegetables	140 (103, 206)	0.06[3]	0.11[3]	0.06[3]	0.10[3]
Legumes	7 (3, 13)	-0.02[3]	-0.07[3]	-0.02[3]	-0.06[3]
Total fruit	239 (120, 340)	0.13[3]	0.11[3]	0.13[3]	0.10[3]
Fresh/canned fruit	187 (99, 282)	0.19[2]	0.16[3]	0.18[2]	0.15[3]
Juice	6 (0, 41)	-0.08[3]	-0.07[3]	-0.09[3]	-0.07[3]
Total dairy	381 (232, 501)	0.03[3]	0.06[3]	0.04[3]	0.07[3]
Reduced fat dairy	277 (44, 446)	0.08[3]	0.08[3]	0.10[3]	0.11[3]
Full fat dairy	9 (2, 22)	-0.06[3]	-0.03[3]	-0.07[3]	-0.05[3]
Total meats and alternatives	169 (105, 217)	-0.15[3]	-0.01[3]	-0.15[3]	-0.01[3]
Red meat	56 (31, 106)	-0.21[2]	-0.04[3]	-0.20[2]	-0.04[3]
Processed meat	19 (8, 32)	0.01[3]	0.02[3]	0.01[3]	0.01[3]
Chicken	34 (19, 51)	-0.16[3]	0.03[3]	-0.15[3]	0.04[3]
Fish	29 (15, 49)	-0.01[3]	0.04[3]	0.01[3]	0.04[3]
Tofu	0 (0, 1)	-0.10[3]	-0.06[3]	-0.09[3]	-0.04[3]
Nuts	4 (1, 10)	-0.17[2]	-0.01[3]	-0.17[2]	-0.02[3]
Eggs	13 (10, 34)	0.07[3]	-0.04[3]	0.06[3]	-0.05[3]
Total extra foods[5]	126 (66, 236)	0.03[3]	0.07[3]	0.04[3]	0.08[3]

[1]$P < 0.01$; [2]$P < 0.05$; [3]$P > 0.05$; [4]Adjusted for age, sex and weight; [5]Takeaways, sweets, savoury snacks and alcoholic beverages. CCA IMT: Common carotid artery intima media thickness.

the cohort had ever smoked and only 5% were current smokers.

Blood pressure, lipids, presence of micro-albuminuria and glycaemic control were not correlated with CCA IMT after age was adjusted for. When subjects that were prescribed a lipid lowering medication were removed from the analysis systolic blood pressure did independently predict CCA IMT. Therefore we may not have detected associations between CCA IMT and blood pressure, lipids, presence of micro-albuminuria and glycaemic control because over 50% of the cohort were prescribed lipid lowering and anti-hypertensive medications and the participants were well controlled in terms of blood pressure, lipid and glucose levels. In this cohort recommendations for blood pressure (< 140/85 mmHg), LDL cholesterol (< 2.5 mmol/L), triglycerides

(< 2.2 mmol/L) and HDL cholesterol (> 1 mmol/L) as defined by the 2013 European Society of Cardiology/ European Association for the Study of Diabetes Guidelines on Diabetes, Pre-diabetes and Cardiovascular Disease were met[26]. In our cohort mean CCA IMT was lower than what has been observed in other populations with diabetes of a similar age. In a meta-analysis involving 4420 individuals with type 1 or type 2 diabetes the mean CCA IMT was 0.79 ± 0.19 mm (mean age 61; IQR = 36-76 years) compared to 0.71 ± 0.13 mm in our cohort (mean age 56 ± 14 years)[11].

The lack of an association between HbA1c and CCA IMT in the present study may be explained by the mean HbA1c (7.3% ± 1.4%) which is lower than what has been reported in studies that have shown a relationship between carotid IMT and HbA1c. Kinouchi *et al*[27] found HbA1c was associated with maximum carotid IMT in a Japanese cohort with type 2 diabetes (n = 167) with a mean HbA1c of 8.3% ± 2.3%. Similarly, Shah *et al*[28] showed a positive relationship between HbA1c and CCA IMT in a cohort of youth with type 2 diabetes (n = 129) with a baseline mean HbA1c of 8.6% ± 3.3%. In the SEARCH CVD study in youth with type 1 diabetes BMI z score was the only modifiable risk factor related to carotid IMT[29].

This study shows that CCA IMT values measured at one time-point are not different between subjects with type 1 and type 2 diabetes, once age is adjusted for, despite the subjects with type 2 diabetes being more metabolically at risk due to higher weight, BMI and triglycerides and lower HDL cholesterol. In contrast to our finding, in a cohort of subjects with newly diagnosed type 1 or type 2 diabetes (type 1: 33 ± 24 d since diagnosis; type 2: 36 ± 21 d since diagnosis) aged 14 to 30 years (mean age type 1: 21 ± 5 years; type 2: 22 ± 5 years) CCA IMT was significantly greater in those with type 2 diabetes[30]. It is likely that the subjects with type 2 diabetes in the study by Gu *et al*[30] had lived with metabolic abnormalities for many years in contrast with the type 1 subjects. In the context of the current study, the finding of the study by Gu *et al*[30] suggests that the duration of type 1 diabetes contributes to carotid IMT. This is supported by a study showing that in subjects with type 1 diabetes, for a mean of 5.5 years, carotid IMT was comparable to healthy subjects 10 years older[31]. Previous research shows carotid IMT progression is similar in people with type 1 (0.036 mm/year)[32] and type 2 diabetes (0.04 mm/year)[33] and significantly greater than the rate of progression observed in non-diabetic people (0.0147 mm/year)[34].

Type 1 and type 2 diabetes are perceived to be different diseases but the risk factors for CVD in both types of diabetes are similar and include insulin resistance, obesity, inflammation, and renal disease[35]. In addition, the burden of CVD is comparable in people with type 1 and type 2 diabetes[3]. Lind *et al*[4] showed that even when people with type 1 diabetes achieve a HbA1c of less than 6.9% (52 mmol/mol) the hazard ratio for death from a cardiovascular cause is 2.92 (95%CI: 2.07-4.13) compared to age and sex matched people without diabetes. In summary, it was found that CCA IMT was not different between people with type 1 and type 2 diabetes once age was accounted for, but it must be acknowledged that a small number (n = 23) of people with type 1 diabetes were included in the study. In the future this finding needs to be investigated in a larger cohort of age matched people with type 1 and type 2 diabetes; ideally a healthy control group would be included to determine whether age has a differential effect in people with diabetes.

In this cohort we observed no association between dietary intake and CCA IMT once age, weight and sex were adjusted for. Subgroup analysis showed that in those that were not prescribed a lipid lowering medication alcohol consumption was positively associated with CCA IMT after multivariate adjustment. Alcohol consumption has been previously shown to correlate with CCA IMT in a healthy Korean population such that in men an inverse relationship was observed but this was attenuated to non-significance after adjustment for lipids; in women a positive association between alcohol consumption and CCA IMT was observed[36]. The Cardiovascular Risk in Young Finns study also showed a positive association between alcohol intake and CCA IMT[37].

Previous observational studies, with a similar sample size to the present study, conducted in populations with type 2 diabetes[38,39] have shown that greater fruit consumption is associated with lower carotid IMT (n = 255) and an inverse association between plasma vitamin C concentration and CCA IMT has been shown in individuals with type 1 diabetes (n = 59)[40]. Curtis *et al*[41] conducted a 1 year randomised controlled trial in post-menopausal women (n = 93) with type 2 diabetes and found that supplementation with 27 g/d of flavonoid-enriched chocolate did not change CCA IMT progression compared to the placebo. A recent meta-analysis of randomised controlled trials showed that lifestyle modification can slow carotid IMT progression[42].

Studies investigating the relationship between carotid IMT and carotenoids including α-carotene, β-carotene, lutein, zeathanin and β-cryptoxanthin have yielded mixed results[43], although there is evidence suggesting an inverse association exists between lycopene and carotid IMT[44]. A recent trial showed that supplementation with lutein (20 mg/d) and lycopene (20 mg/d) reduced CCA IMT progression after 12 mo compared with the placebo treatment in a healthy Chinese population (n = 144)[45]. The authors are not aware of any studies reporting on the relationship between carotenoids and carotid IMT in people with diabetes.

In this analysis there was no association between serum lipid species and CCA IMT. A previous study showed that there was an inverse association between CCA IMT and lysophosphatidylcholine (LPC) 16:0 after adjustment for age and sex (r = -0.13; P = 0.01)[46]. This study also showed that LPC 16:0 and LPC 20:4 were negatively associated with the development of CVD after 12 years of follow-up (LPC 16:0: OR = 0.79; P = 0.028;

LPC 20:4; OR = 0.77; P = 0.024 per standard deviation increase)[46]. Lipid profiling has been shown to discriminate between unstable and stable coronary artery disease (CAD) and it has been suggested that changes in plasma lipids precede the development of plaque instability[17]. Lipidomic analysis can predict the burden of non-calcified coronary artery plaque in asymptomatic patients at intermediate risk of CAD[47]. In the study by Ellims *et al*[47] CCA IMT was not associated with coronary artery plaque burden, although CCA IMT is only weakly correlated with CAD assessed by quantitative coronary angiography[48], despite carotid IMT being correlated with coronary IMT, measured using intravascular ultrasound[49]. Therefore we may not have observed a relationship between CCA IMT and serum lipid species because lipid species are associated with more advanced disease progression.

Limitations of this analysis include the cross-sectional design, small sample size and the use of a FFQ to measure dietary intake, although serum carotenoids were measured as biological markers of vegetable intake. In addition, only CCA IMT was measured and a different result may have been observed if internal carotid artery IMT or bifurcation IMT had been measured as these components are more strongly associated with CAD[50]. Another limitation of this study is that dietary habits were only measured at one point in time and may not be reflective of lifetime exposure, whereas CCA IMT is determined by lifetime exposure, and it is possible that dietary habits or reporting of dietary habits was altered by a range of factors including diabetes diagnosis. The dietary predictors of carotid IMT need to be investigated in a larger cohort as this study may have lacked the required statistical power to detect an effect. Especially since more than half of the cohort was prescribed a lipid lowering or anti-hypertensive medication and this may obscure the relationship between dietary intake and CCA IMT. Power analysis shows that with 120 participants an r value of 0.178 (P < 0.05) or 3% of the variance can be detected so major effects should have been found.

In conclusion, in this cohort of well-controlled individuals with type 1 and type 2 diabetes the strongest predictors of CCA IMT were age and weight, which accounted for 46% of the variance in the model. After multivariate adjustment there were no dietary predictors of mean or mean maximum CCA IMT. However, in subjects that were not prescribed a lipid lowering medication alcohol consumption was positively associated with CCA IMT after multivariate adjustment. There was no correlation between serum lipid species or carotenoids and CCA IMT. There was no difference in mean or mean maximum CCA IMT between subjects with type 1 and type 2 diabetes once age was accounted for but this finding needs to be investigated further.

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COMMENTS

Background

Common carotid artery intima media thickness (CCA IMT) is an early marker of atherosclerosis and predictor of cardiovascular disease (CVD). The dietary predictors of CCA IMT in individuals with diabetes are not well defined. Likewise, there is a lack of data from comparative investigations of CCA IMT in individuals with long duration type 1 and type 2 diabetes.

Research frontiers

Diabetes diagnosis is an important risk factor for CVD, approximately doubling the risk of a cardiovascular event, and this is independent of conventional risk factors including sex, age, smoking status, body mass index and systolic blood pressure. Due to the increasing incidence of diabetes defining strategies to reduce the burden of CVD in people with diabetes is a priority.

Innovations and breakthroughs

After adjustment for age, sex and weight there were no dietary predictors of CCA IMT identified. For the first time is was shown that there was no difference in mean or mean maximum CCA IMT between subjects with long duration type 1 and type 2 diabetes once age was accounted for. This finding needs to be investigated further in a larger cohort of age matched individuals with type 1 and type 2 diabetes.

Applications

The etiology of type 1 and type 2 diabetes are different but the two type of diabetes confer similar CVD risk, which cannot be entirely explained by traditional cardiovascular risk factors. In this study the authors showed that after adjustment for age, CCA IMT was not different in subjects with type 1 and type 2 diabetes. This finding should be explored further.

Terminology

CCA IMT is visualized using B mode ultrasound. The intima-media complex is the area of tissue starting at the luminal edge of the artery and ending at the boundary between the media and the adventitia. CCA IMT is a measure of early atherosclerosis.

Peer-review

This is an interesting study evaluating the predictors of carotid intima-media thickness in patients with diabetes mellitus. The study is well-designed and the results are clearly presented.

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SYSTEMATIC REVIEWS

Barriers that practitioners face when initiating insulin therapy in general practice settings and how they can be overcome

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Abstract

AIM

To explore primary care physicians' perspectives on possible barriers to the use of insulin.

METHODS

This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Eight electronic databases were searched (between January 1, 1994 and August 31, 2014) for relevant studies. A search for grey literature and a review of the references in the retrieved studies were also conducted. Studies that focused on healthcare providers' perspectives on possible barriers to insulin initiation with type 2 diabetic patients were included, as well as articles suggesting solutions for these barriers. Review articles and studies that only considered patients' perspectives were excluded.

RESULTS

A total of 19 studies met the inclusion criteria and were therefore included in this study: 10 of these studies used qualitative methods, 8 used quantitative methods and 1 used mixed methods. Studies included a range of different health care settings. The findings are reported under four broad categories: The perceptions of primary care physicians about the barriers to initiate insulin therapy for type 2 diabetes patients, how primary care physicians assess patients prior to initiating insulin, professional roles and possible solutions to overcome these barriers. The barriers described were many and covered doctor, patient, system and technological aspects. Interventions that focused on doctor training and support, or

IT-based decision support were few, and did not result in significant improvement.

CONCLUSION

Primary care physicians' known delay in insulin initiation is multifactorial. Published reports of attempts to find solutions for these barriers were limited in number.

Key words: Diabetes; Insulin; Initiation; Delay; Barriers; Primary care physicians

Core tip: There are several barriers to primary care physicians in initiating insulin therapy when it is required for type 2 diabetes patients. The main purpose of this systematic review is to explore these barriers in depth. Published reports of attempts to find solutions to these barriers were limited in number. Given the increasing burden of this chronic disease, and the need to optimize and standardize management, it is expected research in this area will remain intense. The research that remains patient-centered and takes a system approach can be expected to yield best results.

Bin rsheed A, Chenoweth I. Barriers that practitioners face when initiating insulin therapy in general practice settings and how they can be overcome. *World J Diabetes* 2017; 8(1): 28-39 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i1/28.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i1.28

INTRODUCTION

Diabetes is a major world health problem. The most recent report from the International Diabetes Federation estimates that 415 million people have diabetes worldwide (8.8% of the adult population). Of these, 193 million people have undiagnosed diabetes, which allows the disease to progress untreated causing many complications. In 2015, diabetes was responsible for 11.6% of total global adult health expenditure and yet there were 5 million deaths from this disease. In addition, the number of people with diabetes is expected to increase to more than 642 million by 2040[1].

Type 2 diabetes is characterized by defects in both insulin secretion (due to a progressive decline in beta cell functioning), and insulin resistance. It is aggravated by obesity and sedentary lifestyle. Untreated hyperglycemia increases the risk of mortality and morbidity, *via* a higher risk of macro-vascular and micro-vascular complications[2-4].

With already high numbers of patients and a relatively small number of diabetes specialists worldwide, 90% of patients receive care for their diabetes from primary care physicians (PCPs)[5,6].

Several trials[7,8] have shown that improving glycemic control, *via* lifestyle modifications and the use of medications, reduces micro-vascular and possibly macro-vascular complications and mortality related to diabetes. However, over time, the progressive nature of beta cell dysfunction results in the inability of oral hypoglycemic agents to control hyperglycemia and achieve HbA1c targets[9]. This gradual loss in beta cell function would indicate that insulin therapy is almost always required at some point to treat diabetic patients[10].

Although most PCPs believe that the initiation of insulin therapy is an essential component in the management of type 2 diabetes, many still consider it to be the "last option" and indicate that their patients are reluctant to accept this therapy. In the seminal Diabetes Attitudes, Wishes, and Needs (DAWN) study, Peyrot *et al*[11] reported that approximately 50% of healthcare professionals delay insulin initiation until it is "absolutely necessary".

Similarly, the SOLVE™ (Study of Once Daily Levemir), a multicenter observational study that involved over 17374 patients with type 2 diabetes in 10 countries (Europe, Asia and North America), showed that insulin initiation is generally delayed until an average HbA1c level of approximately 9%[12]. Several other studies across many countries have confirmed that there is significant delay in the initiation of insulin therapy[13-15]. This reluctance to initiate insulin treatment may be related to patient, provider or system factors.

Reported patient-related barriers include a sense of personal failure, a negative impact on social life, injection phobia, myths and misconceptions about the drug, the permanence of the therapy, difficulties in fulfilling responsibilities at home and at work, limited insulin self-management training, inadequate provider explanation about the risks and benefits of the intervention - and concerns over weight gain and hypoglycemia[16-21]. Most of these negative attitudes and perceptions are described in the literature under the term "psychological insulin resistance". Studies in Various countries have found variable rates of such resistance amongst patients - from 5.9% to nearly 50% of patients[17-25].

System barriers to the use of insulin therapy may include a lack of resources (*e.g.*, staff and materials), a lack of continuity of care, as well as the workload and time constraints of PCPs[26].

As for the providers, part of the problem has been attributed to "therapeutic or clinical inertia; a recognition (by the PCP) of a lack of glycemic control - but a failure to act"[27-29]. These PCPs blame (perceived) patient reluctance, language barriers, their concern for patients' comorbidities and their own lack of training[26].

A few systematic reviews have been published related to patients' perspectives on the delay in insulin initiation or to psychological insulin resistance[30-33]. This study focuses on PCP's perspectives on their barriers to initiating insulin therapy in primary care/general practice. It will also explore the literature for possible solutions.

MATERIALS AND METHODS

This literature search was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-

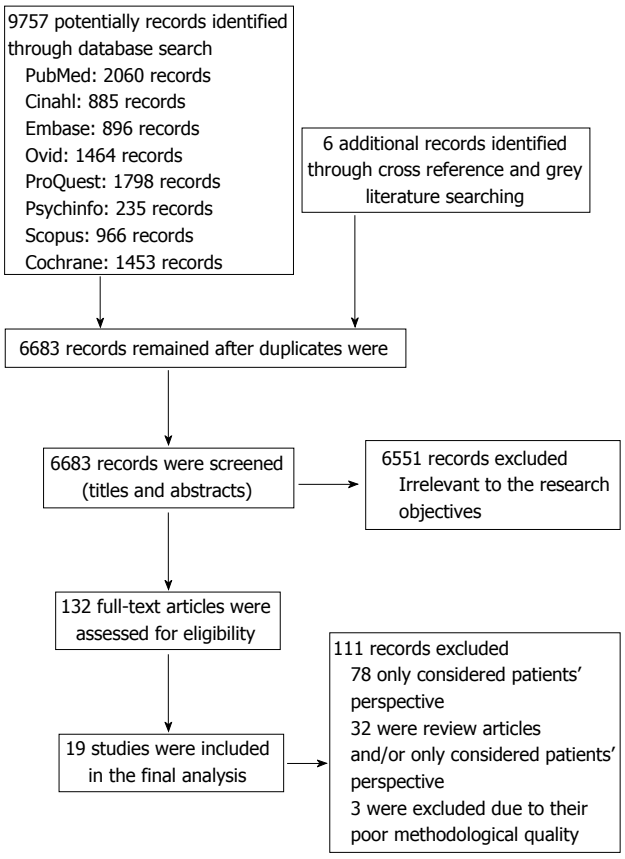

Figure 1 Flow diagram of the studies included in this analysis.

Analyses guidelines[34]. As this research doesn't involve experiments on humans or animals, ethical approval is not required.

A variety of scholarly databases (PubMed, EMBASE, Psychinfo, Cinahl, Ovid, Scopus, ProQuest and the Cochrane library) were searched. The search was conducted using a combination of medical subject headings and text words: Insulin, physician, general practitioner, general practice, primary care, health care, professional, provider, psychological, clinical inertia, attitude, delay, refusal, refuse, perception, postpone, worry, initiation, compliance, knowledge, fear, belief and barrier.

A search in Google and Google Scholar engines were then conducted to look for "grey" literature and the bibliographies of the retrieved articles were also reviewed to identify further relevant studies.

Studies that satisfied the following selection criteria were included: Only original articles, quantitative or qualitative methods, descriptive or analytical, written in English and published between January 1, 1994 and August 31, 2014.

This review was restricted to studies that focused on PCP's perspectives to the barriers they faced regarding the initiation of insulin for type 2 diabetic patients. Articles suggesting solutions for these barriers were also included. Review articles or studies that only explored patients' perspectives were excluded.

The screening and selection of the studies were done independently by the two authors, and the final number of the studies used for this review was reached by consensus between them. The authors assessed the methods used in the various studies: The Critical Appraisal Skills Program checklist[35] was used for the qualitative studies, the STROBE checklist[36] was used for the observational studies, the Mixed Methods Appraisal Tool[37] was used for the mixed-methods studies and the Centre for Evidence-Based Medicine tool[38] was used for the randomized control trials.

The authors had to be in agreement for an article to be included in the systematic review. Both authors also collaborated to extract the data from each article. These are summarized in table form according to authors' name, year of publication, aim of the study, sample size, the setting, instruments used, results and conclusion (Table 1).

RESULTS

The initial search process resulted in 9757 studies. After removing any duplicates and adding articles by searching the cross-references and grey literature, this number dropped to 6683 studies. Titles and abstracts of these studies were then screened, and the two authors narrowed the results down to 132 articles for full-text assessment (78 articles were then excluded because they looked only at patients' perspectives, and a further 32 were excluded because they were review studies).

The remaining 22 articles were critically appraised by the two authors, and three more articles were excluded because of their poor overall quality. Thus, a total of 19 articles were included in the final analysis. Figure 1 is a flow diagram of this process.

Characteristics of the studies

The publication dates of the final 19 studies ranged from 2005 to 2014 and included 10 qualitative studies, 7 observational studies, 1 mixed-method studies and 1 randomised controlled trial.

One study was international and multicentre; three studies were conducted in both the United States and Malaysia; two studies were conducted in the United Kingdom and Australia; and one study was conducted in each of the following countries: Japan, Pakistan, Singapore, South Africa, Israel, Belgium, Canada and Middle Eastern Arab countries. These articles are summarized in Table 1.

The perceptions of primary care physicians

Several researchers tried to explore PCP's barriers to initiating insulin therapy using qualitative methods (*e.g.*, focus group discussions or semi-structured interviews). Hayat *et al*[39], Patel *et al*[40], Lee *et al*[41], Tan *et al*[42] and Haque *et al*[26] conceptualized that the decision to initiate insulin therapy can be influenced by three types of barriers: Physician barriers, patient barriers and system barriers.

From these studies we can say that PCP's barriers may include: A lack of knowledge, training and experience by the PCP; language barriers between PCP

Table 1 Summary of the articles included in this study

Ref.	Design	Study aims	Sample and setting	Tools and outcome measures	Results and conclusions
Hayat *et al*[39] (2010)	Qualitative	To explore barriers and myths regarding initiating insulin therapy in poorly controlled type 2 diabetic patients in primary care centres in Hyderabad District, Pakistan	6 to 20 medical officers per each focus group and 12 medical officers for semi-structured interviews Conducted in primary care centres in the Hyderabad District, Pakistan	6 focus group discussions 12 semi-structured individual interviews	Patients barriers: Mistaken beliefs about insulin; fear of needles and excessive belief in traditional healers Doctors' barriers: Skill and knowledge deficiencies, language barriers and fear of hypoglycaemia and obesity risks System's barriers: High workload, insufficient consultation time, lack of continuity of care and financial barriers
Manski-Nankervis *et al*[51] (2014)	Qualitative	To study the effect of communications and relationships between health care professionals in general practice toward the issue of insulin initiation and to clarify how multidisciplinary teams work in practice	21 GPs, practice nurses, diabetes nurse educators and specialist physicians Conducted in Melbourne, Victoria, Australia	Semi-structured interviews face-to-face or *via* the telephone	Barriers for initiating insulin aren't solely due to medical or training issues; communication and relationships among health care professionals also have a strong influence 4 themes identified from the study: Uncertain roles, unreliable competency, relationships and communication between healthcare workers and the development of trust and respect
Grant *et al*[46] (2007)	Cross-sectional	To study physicians' considerations and preference when selecting medications for patients with type 2 diabetes	886 academic generalists and specialists Conducted in the United States	Questionnaire-based survey	The major considerations for academic generalists when staring insulin are the patient's HbA1c level, adherence and motivation, health assessment and glucose level patterns Major barriers to beginning insulin for generalists are patient derived (patients' fear and preferences), while the specialists didn't specify any major barriers
Yoshioka *et al*[48] (2013)	Cross-sectional	To identify differences in the perceptions of patients and their physicians regarding insulin initiation (using data from the DAWN Japan study)	148 patients with type 2 diabetes and 68 physicians Conducted in Japan	Questionnaire-based survey	There are perceptions gaps regarding insulin initiation between patients and physicians, especially regarding its social impact Physicians tend to overestimate the barrier of the injections being painful and patients' fear and to underestimate its social impact
Peyrot *et al*[11] (2005)	Cross-sectional	To study patients' and healthcare professionals' (physicians and nurses) attitudes toward insulin therapy and its correlation with delaying insulin therapy initiation	2061 type 2 diabetic patients and 1109 providers (physicians and nurses) Conducted in 13 countries in Asia, Australia, Europe and North America	Structured interviews conducted face-to-face or over the telephone	Most healthcare professionals (50%-55%) delay insulin initiation until it is absolutely required Delay in insulin initiation is significantly less likely when providers consider that their patients are adherent to appointments and medication regimens Delay of starting oral hypoglycaemic drugs is the strongest correlation with insulin therapy initiation

Lee *et al*[47] (2014)	Qualitative	To determine how healthcare professionals assess their patients when initiating insulin therapy for type 2 diabetic patients	41 health care professionals (physicians, nurses, diabetic educators and pharmacists) Conducted in Malaysia	4 focus group discussions and 10 individual interviews	Healthcare professionals' assessment of diabetic patients when considering insulin initiation are based on their perceptions rather than objective evaluation of patients' backgrounds, knowledge and abilities
Ratanawongsa *et al*[43] (2012)	Cross-sectional	To explore primary care physicians' perceptions about barriers of initiating insulin for patients with type 2 diabetes	83 primary care physicians Conducted in United States (Indiana, New jersey and California)	Structured interviews that contained open-ended questions	Participants reported that at least 10% of their patients would reject to start insulin 64% of the clinicians believed that their patients' reluctance was the cause of delaying insulin initiation; 43% believed it was due to their patients' poor self-management skills 97% of physicians thought that fear of the injections was the cause of their patients' resistance to starting insulin
Patel *et al*[40] (2012)	Qualitative	To identify healthcare professionals' perspectives on delaying insulin initiation for type 2 diabetic patients in a multi-ethnic setting	14 healthcare professionals (general practitioners, specialists and nurses) Conducted in the United Kingdom	Semi-structured, face-to-face, interviews	Barriers for initiating insulin therapy for South Asian diabetic patients could be over-accentuated by the presence of language barrier and the lack of patients' understanding about the disease and its therapy South Asian patients seem to be more likely to be negatively influenced by observations and experiences about insulin treatment within their community
Lee *et al*[41] (2012)	Qualitative	To explore healthcare professionals' opinions on barriers of initiating insulin therapy in Malaysian multi-ethnic patients with type 2 diabetes	38 healthcare professionals (general practitioners, family medicine specialists, medical officers, policy makers, diabetes educators and endocrinologists) Conducted in Malaysia	Focus group discussions and semi-structured interviews	Patients' barriers: Patients' fear and misconceptions about insulin, lack of knowledge and self-efficacy Healthcare professionals' barriers: Negative attitude toward insulin; lack of training, motivation and confidence System barriers: Lack of continuity of care, shortage of resources and language barriers
Lee *et al*[52] (2012)	Qualitative	To explore the strategies suggested by healthcare professionals to improve insulin initiation in the Malaysian dual-sector (public-private) healthcare system	38 healthcare professionals (general practitioners, family medicine specialists, medical officers, policy makers, diabetes educators and endocrinologists) Conducted in Malaysia	Focus group discussions and semi-structured interviews	Developing an integrated system for patients' referral from the private sector to the public health sector for insulin initiation services Involving nongovernmental organisations, the media and pharmaceutical industry in supporting insulin initiation Establishing multidisciplinary teams
Lakkis *et al*[44] (2013)	Cross-sectional	To investigate family physicians' attitudes towards insulin therapy in type 2 diabetic patients in Middle Eastern Arab countries	122 family physicians Conducted in Middle Eastern Arab countries	Online questionnaire-based survey	73.6% of family physicians chosen to delay insulin initiation until it is absolutely necessary 64% of family physicians reported hesitancy to start insulin mostly due to apparent patient reluctance

					Referral to endocrinologists for initiating insulin therapy was correlated with a lack of experience and concerns about risks, mainly with elderly patients
Nakar *et al*[49] (2007)	Case-control study	To study the barriers that delay a shift to insulin from the perspectives of type 2 diabetic patients and family physicians	Study group: 92 patients who needed insulin Control group: 101 patients who had begun insulin 157 family physicians Conducted in Israel	Telephone interviews with participating patients Written questionnaire obtained from the physicians	A clear perception gap regarding the insulin initiation barriers between patients and physicians Physicians exaggerate the importance of patients' physical fear of pain associated with injections and blood tests, while patient barriers seem to be related more to their concept of the illness
Furler *et al*[50] (2011)	Qualitative	To describe barriers and facilitators to insulin initiation in general practice	14 healthcare professionals (general practitioners and diabetes nurse educators) and 12 type 2 diabetic patients Conducted in Australia	Semi-structured interviews	Insulin initiation could be influenced by the way patients and healthcare professionals Interact There was a disagreement and uncertainty about the healthcare workers' role in initiating insulin
Hayes *et al*[45] (2008)	Cross-sectional	To explore primary care physicians' attitudes toward insulin initiation for type 2 diabetic patients and to determine the areas where there is a clear lack of consensus between them	505 primary care physicians Conducted in the United States	Online questionnaire-based survey	Highest consensus was attitudes related to risk and benefits of insulin therapy, positive experiences of diabetic patients on insulin and patient worries about insulin initiation Clear lack of consensus was seen in attitudes related to the metabolic effects of insulin, the necessity for insulin therapy, the duration needed for training and the fear of hypoglycaemia risk especially in elderly patients
Tan *et al*[42] (2011)	Qualitative	To explore the barriers to insulin initiation for diabetic patients managed in primary care polyclinics in Singapore	18 healthcare professionals (physicians and nurses) and 11 type 2 diabetic patients Conducted in Singapore	Focus group discussions	Patient barriers to insulin initiation were denial the need for insulin therapy, perception of social stigma, inconvenience, worries of needles pain, fear from side effects and complications Physician attitude and experience with insulin therapy were also a possible barrier
Haque *et al*[26] (2005)	Qualitative	To explore the barriers to initiating insulin therapy for type 2 diabetic patients in public-sector primary health care centres in Cape Town, South Africa	46 medical officers working at community health centres Conducted in Cape Town, South Africa	5 focus group discussions and 10 individual semi-structured interviews	Physician barriers: Lack of knowledge and experience, language barriers and exaggerated fear of hypoglycaemia Patient barriers: False beliefs about insulin, poor compliance, lack of understanding of the disease, belief in traditional herbs, fear of injections and poor socioeconomic status System barriers: Time limitations, lack of continuity of care and financial restraints

Sunaert *et al*[53] (2014)	Qualitative	Related to a program supporting the initiation of insulin therapy in primary care in Belgium, this study determined factors influencing the general practitioners to be involved in insulin therapy initiation and explored factors relevant for future program development	9 general practitioners for focus group discussions 20 general practitioners for individual interviews 10 type 2 diabetic patients for individual interview Conducted in Belgium	Focus group discussions Individual semi-structured interviews	General practitioners whom engaged in insulin initiation program differ from those not engaged in: Attitude, subjective norm and perceived behavioural control regarding insulin initiation Factors to consider include: Job boundaries between the diabetes nurse educators and general practitioners, job boundaries between general practitioners and specialists and protocol adherence
Burden *et al*[54] (2007)	Mixed	To evaluate the "Insulin For Life" training course for general practitioners and practice nurses in the Heart of Birmingham Teaching Primary Care Trust by exploring the attitudes of the patients, nurses and GPs toward initiating insulin therapy	39 type 2 diabetic patients using a questionnaire 3 to 6 mo after starting insulin 17 general practitioners and practice nurse surveyed using a questionnaire after course completion 37 GPs and practice nurses attended focus group discussions Conducted in Birmingham, United Kingdom	Questionnaire-based survey Focus group discussion	Post-course completion: Type 2 diabetic patients reported that starting insulin in general practice is acceptable and were confident and self-management Most general practitioners and practice nurses were confident about initiating insulin
Harris *et al*[55] (2013)	Randomised control trial	To determine the effectiveness of an insulin initiation strategy utilising a diabetes specialist and community retail pharmacy support to increase family physician insulin-prescribing rates	73 family physicians in the intervention group were provided with diabetes specialists/educators consultation support for 12 mo and community retail pharmacist support 78 family physicians in the control group had usual care Conducted at 15 sites across Canada	Primary outcome was insulin-prescribing rate (IPR) per physician defined as the number of insulin starts of insulin-eligible patients during the 12-mo period	No significant differences were found between the two groups: Mean IPR of 2.28 compared to 2.29 for Intervention group physicians and the control group physicians, respectively. And an estimated adjusted RR (95%CI) of 0.99 (0.80 to 1.24); $P = 0.96$ An insulin initiation support program utilising support from diabetes specialists, diabetic educators and community retail pharmacists to improve insulin prescribing in family practice was unsuccessful

DAWN: Diabetes Attitudes, Wishes, and Needs

and patient; accentuated concern by the PCP about the risk of hypoglycemia and weight gain; and PCP concerns about patient noncompliance.

In one of the DAWN studies, Peyrot *et al*[11] found that a delay in beginning insulin therapy is significantly less frequent among specialists in comparison to PCPs and that healthcare providers (PCPs, specialists and nurses) usually underestimate a patient's feelings of self-blame and personal failure when insulin is needed.

In another study, Ratanawongsa *et al*[43] reported that 64% of PCPs cited patients' resistance as the major cause for delaying insulin initiation, and 97% of these physicians believed that this resistance was due to a fear of injections.

In Arab countries, 73% of PCPs prefer to delay insulin therapy until it is "absolutely essential"[44]; this percentage is higher than the data from the DAWN report (*i.e.*, 50% to 55%)[11].

In an earlier study in the United States, Hayes *et al*[45] explored the attitudes of 505 PCPs in the United States regarding initiation of insulin. Most reported that initiation of insulin is the most difficult part of managing type 2 diabetes, due to the need for injections.

How PCPs assess patients prior to initiating insulin

The way in which PCPs assess their diabetic patients could highly impact their decision to start insulin. Grant *et al*[46] surveyed 886 academic PCPs and specialists in the United States. They reported that when initiating insulin, PCPs usually consider their patients' HbA1c levels, adherence, motivation to improve, overall health assessments and blood glucose level patterns. Patients' age, weight and hospital protocol or guidelines had less impact on their decisions. They appeared to consider

patient concerns in their assessments of barriers, whereas few specialists pointed to any major barriers in insulin initiation at all[46].

In a similar approach a qualitative study conducted in Malaysia of over PCPs explored what assessment they made of patients requiring insulin. They concluded that the decision to start insulin therapy was influenced by assessments of their patients' characteristics, not just their HbA1C or pattern of random glucose readings[47].

Two studies discussed the existence of a perception gap between physicians and patients when initiating insulin. Physicians tend to underestimate the social issues related to insulin usage, they also tend to underestimate patients' understanding of the illness, including concerns that the disease will worsen, self-blame and feelings of personal failure in controlling disease progression. Rather, physicians seemed to overestimate patients' fear and concerns that the injections will be painful[48,49].

Professional roles

The initiation of insulin could be influenced by the way that patients and healthcare providers interact. The impact of roles, communication and the relationships between healthcare professionals and their patients was explored in two qualitative studies conducted in Australia.

Furler *et al*[50] interviewed 14 general practice healthcare providers and 12 patients with type 2 diabetes and concluded that there was uncertainty regarding whose role it was to initiate insulin, *e.g.*, the patients were unclear if the diabetes nurse or diabetes educators were allowed to initiate insulin therapy or whether they only had support roles. The study concluded that there were differing perceptions of what is to be done, who does it, how it is done, and how it is supported. To quote: "…initiating insulin for the treatment of diabetes in the setting of general practice is a complex social intervention".

Manski-Nankervis *et al*[51] interviewed 21 PCPs, practice nurses, diabetes nurse educators and specialists. Both specialists and PCPs agreed that insulin initiation can be undertaken by general practitioners supported by diabetes educators, with specialists backing them up for the complicated cases. They found (again) concerns regarding the ambiguous roles and involvement of nurses (especially practice nurses *vs* diabetes nurse educators) in insulin initiation. However, there was a general feeling that nurses could play a vital role by providing specific training and education[51].

Possible solutions

Our review found four studies that explored solutions to the barriers to insulin initiation. In one study, healthcare professionals and health policy makers in Malaysia were interviewed and participated in focus group discussions. The participants concluded that there is a need to establish multidisciplinary teams and to develop an integrated system for collaboration between the private and public sectors, particularly regarding insulin initiation programs, to decrease the workload (especially of the public sector). The participants also emphasized the importance of the involvement of nongovernmental organizations, the media and the pharmaceutical industry in insulin initiation programs through the provision of training, education and financial support[52].

In another qualitative study conducted in Belgium, PCPs were invited to attend a support program addressing barriers to insulin initiation; this program involved education and specialist coaching. Comparing PCPs who participated with those who did not, the authors analyzed their findings in terms of the theory of planned behavior. This social cognition model includes consideration of attitude, subjective norm and perceived behavioral control when exploring determinants of professional behavior. Compared to PCPs who did not participate in the program, PCPs who participated noticed the following changes in their behaviors: They were more satisfied with their jobs, more interested in becoming involved in insulin initiation, felt strengthened by the appreciation of their patients and had higher self-esteem levels due to their roles in diabetes care being acknowledged by health policy makers[53].

Similarly, a 2007 study conducted in the United Kingdom to partially evaluate a training course for insulin initiation found that most of the healthcare providers thought that the course was useful and made them more confident in dealing with diabetic patients[54].

Finally, Harris *et al*[55] in 2013 reported a randomized controlled trial of over 151 PCPs in 15 locations across Canada to determine the effectiveness of a 12-mo insulin initiation strategy within primary care. This strategy included diabetes specialists and community retail pharmacists supporting PCPs in their initiation of insulin therapy. The primary outcome they measured was insulin prescribing rates. Surprisingly, their results showed no significant difference when using this strategy; the mean insulin prescribing rates for the intervention group PCPs and the control group PCPs were 2.28 and 2.29 respectively. The authors suggested these results might be influenced by some factors: It may be underpowered by having a smaller than estimated sample size, or the possibility of contamination (although it was limited) or the delay of recruitment and participation of the pharmacists. In addition, they considered the "Hawthorne" effect could be operating (where physicians tend to improve their behavior in response to their knowledge of being observed); and that this could explain some of the lack of difference between the two groups.

DISCUSSION

To the best of our knowledge, the present study is the first systematic review of published work that has explored primary care physicians' points of view regarding the noticeable delay in insulin therapy initiation in primary care settings.

This clinical inertia affects approximately one-third of patients with type 2 diabetes, and this failure of

Table 2 Barriers reported by primary care physicians to initiate insulin

Physicians' related barriers
PCP's lack of knowledge, training and experience
Language barriers between PCP and patient
PCP's concern over the risk of hypoglycemia and weight gain
Perceived patient resistance - esp. fear of injections
PCP's concern about patient noncompliance
System barriers
Lack of resources (*e.g.*, staff and materials)
Lack of continuity of care
The workload and time constraints of PCPs
Ambiguity of roles in the primary care team

PCP: Primary care physician.

PCPs to act, coupled with patients' prolonged exposure to hyperglycemia, leads to a higher risk of chronic complications and mortality[28].

Our review finds that PCPs report many barriers to the initiation of insulin and these range across doctor, patient and system issues. Others have also pointed to this complexity[26,39,41,51]. Table 2 listed the main barriers reported by PCPs to initiate insulin as found in this review.

Clearly, the knowledge, training and experience of the PCP is an important factor, and must affect patient management in more domains than just insulin initiation[11]. This factor may also overlap with an over-concern (by the PCP) about their patient's ability to cope with injections or about possible patient side-effects such as weight-gain and hypoglycemia[26,39,42-44].

Less obviously connected to training and experience is the finding that PCPs under-estimate their patient's feelings of guilt and personal failure when faced with insulin initiation. About 40% of diabetic patients report poor psychological well-being; guilt and self-blame may well be included in this[56].

And PCPs may assess their insulin-requiring diabetic patients in ways different to their specialist colleagues - considering patient adherence and motivation (as well as general health status, HbA1C and blood glucose levels)[46,47]; thus delaying insulin initiation. Yet a concern for this wider assessment seems to miss relevant patient concerns such as the impacts (of insulin use) on their social, marital, occupational or financial situations, as well as causing inconvenience and social stigma. Patients also report lack of understanding of the illness and the role of insulin[48,49]. Perhaps PCPs hear this patient reluctance and assume it is mainly about the use of injections.

Counter to this is the reassuring findings of two studies that suggest patients' negative attitudes toward insulin are mostly temporary and improve after they begin using insulin[57,58]. Perhaps patients need education and reassurance earlier in their diabetic journey; that insulin will most probably be needed at some stage, despite their best efforts, and that this is part of the natural history of the disease - not a sign of their poor management and certainly not a reason for self-blame.

There have been various opinions and suggestions for improving the timeliness of insulin initiation. These have included better delineation of roles within the primary health care team, better integration of health care services, improved communications within the health care setting, improved education of patients (including in groups), wider use of nursing staff, decision support for PCPs, or harnessing new technologies[52-55]. These are all consistent with the suggested approach captured within the Chronic Care Model[59].

Some of the above ideas have attracted intervention studies, with varying success and/or methodological rigor: Some interventions have targeted the PCP (including how to deal with barriers to insulin initiation) but there are few in the literature that report clinically positive effects. Two have evaluated such interventions and found that PCPs reported "increased confidence" in using insulin therapy[53,54].

But with a definite clinical outcome measure (HbA1C over three years), Dale *et al*[60] demonstrated a sustained effect from a brief training course on insulin initiation for healthcare professionals. The training involved "presentations, small group work, case studies and practical demonstrations".

On the other hand, Harris *et al*[55] (in a randomized controlled trial) could not demonstrate any clinical effect from an intervention that provided external expert consultation support to PCPs by community pharmacists and specialists.

Another controlled trial used a decision-support tool aimed at helping PCPs make decisions about insulin therapy. They found no difference in insulin initiation between control and intervention groups - though physicians were allowed not to consult the decision support tool[61].

Clearly, interventions aimed at the PCP will need further refinement, especially if they are to be scaled up for the future increasing numbers of diabetic patients.

Of course technology may change the discourse about clinical inertia or psychological insulin resistance in other ways. For example prefilled insulin pens could reduce the time needed for patient education and training. This method is associated with increased patient convenience and persistence[62]. Other technical developments can also be expected. Table 3 listed the possible solutions for insulin initiation delay proposed in the literature.

Limitations

This systematic review concentrated only on studies of Primary Care Physicians.

It integrates the findings from a variety of research designs; thus a meta-analysis couldn't be used. Sampling varied greatly amongst the studies: Some included patients as well as healthcare providers (even health policy experts), and some didn't report how the subjects were recruited. Moreover, the research was restricted by the use of articles written in English, which could miss some studies related to this review subject.

In conclusion, PCPs' known delay in insulin initiation

Table 3 Possible solutions for insulin initiation delay proposed in literature

Establish multidisciplinary teams and an integrated system for insulin initiation programs
Involve non-governmental organisations through provision of training and education for both patients and healthcare workers
Reinforce healthcare workers skills and knowledge through training courses and workshops
Consider Involving clinical pharmacists and specialists in insulin initiation programs as kind of support or backup
Consider using the aid of electronic decision-support tool
Adopting technology like prefilled insulin pens

is multifactorial, but our understanding of these is getting better, *e.g.,* perceptions of PCPs about patient reluctance may be missing important details such as patient guilt and self-blame. Upskilling of PCPs remains an attractive approach, while the particular elements of teamwork, clinic processes, clinic resources and decision support will need further study and refinement.

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COMMENTS

Background

The reluctance of many general practitioners to initiate insulin treatment could be related to patient, provider and/or system factors. The purpose of this study is to explore general practitioners' perspectives on possible barriers to the use of insulin therapy and to look for solutions to this dilemma in the current literature.

Research frontiers

A few systematic reviews have been published previously related to patients' perspective on the delay in insulin initiation or to psychological insulin resistance. What is unique in this study is the focus on primary healthcare providers' perspectives on barriers to the initiation of insulin therapy in primary care/general practice, and the investigation of possible solutions.

Innovations and breakthroughs

There are several barriers to primary care physicians initiating insulin therapy when it is required. Of these, patients' perceived reluctance seems to be a prominent factor, though it is only one of many aspects of this complex issue.

Applications

Published reports of attempts to find solutions to these barriers were limited in number. Given the increasing burden of this chronic disease, and the need to optimize and standardize management, it is expected research in this area will remain intense. The research that remains patient-centered and takes a systems approach can be expected to yield best results.

Terminology

Clinical inertia could be defined as the failure of healthcare provider to initiate or intensify therapy when it is clinically required.

Peer-review

In this systematic review, the authors tried to explore primary care physicians' perspectives on possible barriers to the use of insulin therapy and to look for solutions to this dilemma in the current literature. The authors concluded that primary care physicians' known delay in insulin initiation is multifactorial. Published reports of attempts to find solutions for these barriers were limited in number. This is an interesting systematic review.

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EDITORIAL

Diabetic ketoacidosis: Treatment in the intensive care unit or general medical/surgical ward?

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Abstract

Diabetic ketoacidosis (DKA) is defined as an acute metabolic disorder, which is characterized by an increased presence of circulating ketones, and the development of ketoacidosis in the presence of hyperglycemia. This syndrome occurs as a result of insulin deficiency. Patients can be dramatically ill, however, with aggressive treatment, most patients recover rapidly. Despite being a low-risk condition, the development of acidosis, is one of the admission criteria to the intensive care unit (ICU) for these patients, in order to provide close monitoring, and recognize complications that could result from the use of aggressive therapy, such as continuous infusions if insulin. In some institutions, DKA is treated in the emergency department and general medical/surgical wards to avoid ICU overcrowding.

Key words: Diabetic ketoacidosis; Diabetes; Hyperosmolar non-ketotic state; Clinical outcomes; Serum ketones

Core tip: Diabetic ketoacidosis is a complication for some patients with insulin-dependent diabetes mellitus as well as for non-insulin dependent. It is treated commonly in the intensive care unit (ICU), even though clinical data from many studies support management in regular (medical/surgical) wards, avoiding expensive critical care unit costs and preventing bed crisis in these higher level of care units for sicker patients. Once the patient is treated, adequate follow up and education is mandatory. Noncompliance remains the primary concern for repeated admissions.

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INTRODUCTION

Patients with diabetes mellitus (DM) have health care costs 2.3 times higher than others without this diagnosis[1]. In a prevalence-based study, by the American Diabetes Association, in the United States in 2012, the total cost for diagnosed DM was $245 billion United States dollars, and of it, $176 billion was used for direct medical care costs[1]. In addition, and even more concerning, is the fact that hospitalizations for patients with DM have being increasing[2]. The National Surveillance of Diabetes Public Health Resources, reported that diabetic ketoacidosis (DKA) admissions increased from 80000/year in 1988 to 140000/year in 2009[2].

DKA causes an acute metabolic disorder, which is primarily characterized by an increased presence of circulating ketone bodies, and the development of severe ketoacidosis in the presence of prolonged uncontrolled hyperglycemia, usually due to insulin deficiency[3]. It is more commonly seen in patients with insulin-dependent diabetes mellitus (IDDM), especially among children and young adults. Occasionally, patients with insulin resistant DM can present this complication; especially those that are noncompliant with insulin therapy or who present severe infection[3]. DKA has arbitrarily been classified by some as mild, moderate and severe, according to the initial diagnostic criteria (which includes plasma glucose, arterial pH, serum bicarbonate, urine and serum ketones, serum osmolality and anion gap; and the alteration in the mental status)[4].

EPIDEMIOLOGY

In 2012, 29.1 million Americans or 9.3% of the population were estimated to suffer from DM, according to the American Diabetes Association and the Center for Disease Control and Prevention[2]. Of them, approximately 1.25 million American children and adults have IDDM. This clinical condition has a cumulative incidence of 1.4 million Americans per year and it remains the 7th leading cause of death in the United States since 2010[2]. As noted above, the number of cases of DKA has steadily increased over the past 2 decades[2,3]. In one study in the United States, DKA presentations to the emergency department (ED) increased 35% from 1996 to 2006[3]. When compared to other countries like England, Austria and Germany, the United States has the highest rates of DKA in children with IDDM[5]. Mortality rates for patients with hyperglycemic syndromes (DKA and hyperosmolar non-ketotic states) have been reported as 0.02% in patients with diabetes who are 45 years or younger, and 0.014% among older adults[6]. In some studies, the average length of stay in the hospital for patients with DKA has decreased from 5.7 to 3.4 d, being longer for patients categorized in the "severe" group[2,7]. In the authors' experience, some patients can even be discharged within 23 h of hospital admission despite an initial severe acidemia.

IS DKA A CRITERION FOR ICU ADMISSION?

In many institutions, and for decades, DKA has been routinely treated in ICU environments, including recommendations by the American Diabetes Association guidelines for DKA treatment[3,4,7-9]. The primary reason for these level of care requirements, has been the presence of severe metabolic acidosis, even if patients are grouped as mild or moderate in severity[10]. Frequent blood glucose monitoring, the need for intravenous insulin infusions, and the requirement of frequent vital signs is cited as the hospital structural requirements for this ICU level of care[11]. However, several studies have shown that DKA can be safely treated in the ED or even in medical wards (Table 1)[12-17]. By taking this lower level of care approach, we can potentially avoid ICU hospitalization rate and higher costs, bed overcrowding and reserving the beds for patients who present complications such as hypotension, coma, acute myocardial ischemia, or those with several comorbidities (*i.e*., end-stage renal disease, congestive heart failure) and anyone categorized as suffering severe DKA[12,18,19]. In some observational studies DKA patients admitted to the ICU have a shorter length of stay when compared to non-diabetic mellitus ICU patients[20,21]. A recent retrospective cohort study of 156, 842 hospitalizations among 94 acute-care hospitals, analyzed the adjusted cost of hospitalizations in lower and higher ICU utilizations groups, and concluded that the overuse of ICU only increases the cost and the utilization of invasive procedures but with no improvement in hospital mortality[22].

In a prospective, randomized clinical trial in India, Karoli and coworkers reported that once the DKA patient is evaluated in the ED, and categorized in the severity score, direct admission to a regular ward provided no additional mortality and the only complication noted was hypoglycemia. Other groups have used other classifications to allocate resources for patients with DKA[15]. In a retrospective study, Marinac and Mesa, using laboratory criteria (serum bicarbonate, anion gap, base excess and serum osmolality), and diastolic blood pressure, patients were grouped in 5 grades (Grade 0 - Ⅳ)[19]. ICU admission was recommended only for those who had grade Ⅳ DKA[19] (Table 2).

TREATMENT OPTIONS IN THE ED OR ICU

The treatment of acute DKA includes restoration of fluid

Table 1 Clinical trials comparing care in the intensive care unit *vs* the emergency department or medical ward for patients with diabetic ketoacidosis

Ref.	Country	Patients enrolled	Site of management	Therapy used	Outcome	Length of stay
Dunbar *et al*[12] Retrospective study (January 1994 - March 1995)	United States	61	15: ICU 46: Regular floor	Not mentioned	Mortality due to sepsis in only 1 patient with initial pH < 7.00	ICU: 2 d Regular floor: Not mentioned
Umpierrez *et al*[14] Prospective randomized open trial	United States	45	15: ICU 30: ED	ICU: Intravenous insulin drip ED: 15 subcutaneous insulin aspart Q1H ED: 15 subcutaneous insulin aspart Q2H	Hypoglycemic event presented in each group in only 1 patient per group. No complications, no recurrence of ketoacidosis and no mortality	ICU: 4.5 ± 3 d ED with SC Q1H: 3.4 ± 3 d ED with SC Q2H 3.9 ± 3 d
Karoli *et al*[15] Prospective randomized open trial (January 2009 - June 2010)	India	50	25: ICU 25: ED	ICU: 25 intravenous regular insulin ED: 25 subcutaneous insulin lispro	Hypoglycemic event presented, 2 patients in the ICU group and 1 patient in the ED group. No complications, no recurrence of ketoacidosis and no mortality	ICU: 6.6 ± 1.5 d ED: 6.0 ± 1.2 d
Ersöz *et al*[16] Prospective randomized open trial	Turkey	20	20: ICU	ICU: 10 intravenous regular insulin ICU: 10 subcutaneous insulin lispro	No need to switch to IV regular insulin, no hypoglycemic events, no complications, no recurrence of ketoacidosis and no mortality	Not mentioned
Umpierrez *et al*[18] Prospective randomized open trial	United States	20	10: ICU 10: MW	ICU: 20 intravenous regular insulin IMU: 10 subcutaneous insulin lispro Regular floor: 10 subcutaneous insulin lispro	Hypoglycemic event presented in each group in only 1 patient per group, no complications, np recurrence of ketoacidosis and no mortality	IMU and Regular floor: 4 ± 2 d ICU: 4 ± 1 d
Sotiropoulos *et al*[25] Prospective study (June 2007 - May 31 2008)	Greece	21	21: ED	ED: 21 intravenous regular insulin	Myocardial infarction in only 1 patient - Mortality 4.7%	Not mentioned
Della Manna *et al*[26] Controlled clinical trial (June 2001 - June 2003)	Brazil	60	3: ICU 57: ED	ICU: 3 intravenous regular insulin ED: 27 intravenous regular insulin ED: 30 subcutaneous insulin lispro	Hypoglycemic event on 10 patients, 6 patients due to regular insulin and 4 due to lispro; no complications, no recurrence of ketoacidosis and no mortality	Not mentioned

IMU: Intermediate care unit; SC: Subcutaneous; Q1H: Every hour; Q2H: Every two hours; ICU: Intensive care unit; ED: Emergency department; MW: Medical ward; DKA: Diabetic ketoacidosis.

Table 2 List of conditions requiring admission of patients with diabetic ketoacidosis in the intensive care unit

Myocardial infarction
Congestive heart failure
Acute renal failure
Acute respiratory failure
Altered mental status
Coma
Shock
Hypothermia
Sepsis
Pancreatitis
Gastrointestinal bleeding
Uncontrolled hypertension
End stage renal disease
Hyperkalemia

deficits in the first 24 to 36 h, electrolyte replacement and insulin therapy, which is administered slowly to decreased plasma glucose[23,24]. As noted above, a few randomized, open label trials have proved good outcome and non-inferiority for patients who are managed on regular medical/surgical wards while using with rapid acting insulin, aspart or lispro[13,15,17,25-29].

By establishing a rapid diagnosis and starting treatment in the ED, clinicians can help patients to decrease their costs and hospital stay.

The primary issue in patients with DKA remains the need for repeated hospital admissions. Non-compliance in these patients makes the outcome and prognosis worst. Indeed, medical non-compliance and adherence to the outpatient treatment is the most common precipitating factor leading to the development of moderate-to-severe DKA, requiring ICU admission secondary to complications (*i.e.*, cerebral edema, sepsis) and making the management in the ED and/or ICU very complex[21,25,30]. Life-support care, such

as mechanical ventilation, vasopressors, intravenous antibiotic therapy and mortality rates are higher in these patients, when compared to patients not requiring these interventions[30].

CONCLUSION

The benefit of ICU level of care for patients with DKA rather than regular medical/surgical wards is not well established for patients with mild-to-moderate DKA. Many studies suggest the utilization of the ED or the regular (medical/surgical) wards in the management of these patients. There is significant cost-benefit in managing DKA in the ED and regular wards instead of the ICU, where only patients that require life-supportive intervention should go. Once patients are discharged from the hospital adequate follow up is necessary to avoid readmissions and assure compliance.

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REVIEW

Integrating insulin-like growth factor 1 and sex hormones into neuroprotection: Implications for diabetes

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Abstract

Brain integrity and cognitive aptitude are often impaired in patients with diabetes mellitus, presumably a result of the metabolic complications inherent to the disease. However, an increasing body of evidence has demonstrated the central role of insulin-like growth factor 1 (IGF1) and its relation to sex hormones in many neuroprotective processes. Both male and female patients with diabetes display abnormal IGF1 and sex-hormone levels but the comparison of these fluctuations is seldom a topic of interest. It is interesting to note that both IGF1 and sex hormones have the ability to regulate phosphoinositide 3-kinase-Akt and mitogen-activated protein kinases-extracellular signal-related kinase signaling cascades in animal and cell culture models of neuroprotection. Additionally, there is considerable evidence demonstrating the neuroprotective coupling of IGF1 and estrogen. Androgens have also been implicated in many neuroprotective processes that operate on similar signaling cascades as the estrogen-IGF1 relation. Yet, androgens have not been directly linked to the brain IGF1 system and neuroprotection. Despite the sex-specific variations in brain integrity and hormone levels observed in diabetic patients, the IGF1-sex hormone relation in neuroprotection has yet to be fully substantiated in experimental models of diabetes. Taken together, there is a clear need for the comprehensive analysis of sex differences on brain integrity of diabetic patients and the relationship between IGF1 and sex hormones that may influence brain-health outcomes. As such, this review will briefly outline the basic relation of diabetes and IGF1 and its role in neuroprotection. We will also consider the findings on sex hormones and diabetes as a basis for separately analyzing males and females to identify possible hormone-induced brain abnormalities. Finally, we will introduce the neuroprotective interplay of IGF1 and estrogen and how androgen-derived neuroprotection operates through similar signaling cascades. Future research on both neuroprotection and diabetes should include androgens into the interplay of IGF1 and sex hormones.

Key words: Diabetes; Androgens; Estrogen; Insulin; Insulin-like growth factor 1; Neuroprotection; Brain

integrity; Cognition

Core tip: Insulin-like growth factor 1 (IGF1), estrogen, and androgens are known to have neuroprotective properties. Fluctuations in these hormones is observed in patients with diabetes, varies with sex, and may contribute to abnormalities in brain integrity and cognitive impairment typical of the disease. While the neuroprotective coupling of estrogen and IGF1 has been studied extensively, little research has focused similarly on androgens. Furthermore, research investigating the IGF1-sex hormones relation to diabetes and brain-health outcomes is minimal. One avenue of approach to extend this literature may be to examine sex differences by comparison of these hormone levels, brain integrity, and cognitive aptitude.

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INTRODUCTION

Diabetes mellitus is a metabolic syndrome known for impaired insulin production. This condition is associated with an abundance of sequelae including cardiovascular disease[1,2], brain atrophy[3,4], and more recently, Alzheimer's disease[5-7]. Over the past thirty years, researchers have established strong evidence supporting a link between patients with diabetes and subsequent cognitive impairments and abnormalities in brain integrity.

While meta-analyses have found inconsistencies in the specifics of the literature[8-10], general trends point to cognitive impairments and abnormalities in related structural and functional brain areas. For example, patients with type 1 diabetes (T1D) are frequently found to have decreased psychomotor speed, mental flexibility, and IQ scores[8,11-13]. T1D patients also often show reductions in the volume of regional gray matter in areas such as the prefrontal cortex, hippocampus, and thalamus[12,14,15]. On the other hand, affected skills in type 2 diabetes (T2D) are largely executive function, memory, and information processing[16,17]. Neuroimaging studies done on T2D patients indicate global brain atrophy and microstructural changes[4,7,9,18], while findings regarding white matter hyperintensities are mixed[3].

In both T1D and T2D these decrements are considered mild across most age groups[8,11,19]. The severity of cognitive impairments and brain abnormalities are correlated with age of onset in T1D[11] and duration of the disease in T2D[20,21]. Age is also a risk factor as deficits in learning and memory have been reported to worsen considerably in T2D patients above 65 years of age[22]. Findings suggest the decreased brain volume in patients with T2D is correlated with increased insulin resistance[23], and both brain atrophy and microstructural changes are associated with impaired cognitive performance[18,20].

These data lend support to the idea that brain integrity is compromised in patients with both T1D and T2D, but also emphasize the need to integrate peripheral biomarkers associated with neuroprotection into diabetes research in humans. Various hormones altered as a result of diabetes have been recognized as neuroprotective, including insulin-like growth factor 1 (IGF1) and sex hormones. Research has revealed differences in the serum levels of IGF1 and gonadal hormones in diabetic patients[24-27], with clear sex differences in the effects of androgens and estrogens on the brain in animal models[28].

There is currently a movement in biomedical research to incorporate analyses of sex differences into studies[29-31]; however, studies on brain integrity of diabetic patients often fail to examine men and women separately. This is despite findings of sex-specific differences in regional brain volume between men and women[32-34]. For instance, DTI scans have also reported white matter hyperintensities are different in men and women diabetics[35]. Others have shown that, by combining the data of men and women, T2D patients had smaller gray matter volume with larger ventricular volume and white matter lesions compared to healthy controls. However, when the sexes were analyzed separately, the data for men failed to reach statistical significance[36].

Because sex hormones can act on similar molecular pathways as IGF1, and IGF1 is functionally related to insulin and diabetes, there is a need to further investigate how these hormones interact in the brains of diabetic patients. The relationship between estrogen and IGF1 is the most extensively studied in the neuroprotection literature[37-39], but it has yet to expand experimentally into diabetes research. Furthermore, little attention has been paid to androgen-IGF1 interactions, even in the animal literature, despite the similar mechanisms underlying estrogenic and androgenic neuroprotection.

DIABETES AND IGF1 RELATION

IGF1 has a hypoglycemic response similar to insulin and, in some circumstances, is capable of modulating insulin receptor (IR) activities. Research has demonstrated that low IGF1 is associated with T1D and T2D[40-42]. Moreover, genetic studies suggest decreased IGF1, due to a genetic polymorphism in the promoter region of the IGF1 gene, increases the risk of glucose intolerance and T2D[43].

On the other hand, T2D has also been correlated

with excessively high levels of IGF1. For example, people with acromegaly - a condition known for its overproduction of pituitary growth hormone - have both high levels of IGF1 and a greater risk of developing T2D[44]. These findings were corroborated by two large studies from Denmark (n = 3354) and Germany (n = 7777) which found U-shaped associations between IGF1 levels and the likelihood of developing insulin resistance and T2D[24,25]. Moreover, treatment with IGF1 can improve glycemic control in patients with T1D and T2D[45,46], which may suggest an optimal range of IGF1 for normal glycemic control.

Although IGF1 is synthesized in the brain, peripheral values cannot be used to accurately infer brain levels of IGF1 in humans as local synthesis of IGF1 in the brain appears not to correlate with the quantity of IGF1 receptors (IGF1R)[47-49]. Evidence from animal models suggest that brain atrophy and loss of DNA are prevented following injection of insulin and IGF1, but not insulin alone, into cerebrospinal fluid of mice[50]. Thus, proper systemic levels of IGF1 and its transport from the periphery into the brain is likely necessary for the maintenance of various cognitive processes[51].

Collectively, these data support the involvement of IGF1 in diabetes but also point to an "optimal range" of IGF1. Future research should examine the significance of an optimal peripheral range in the development and maintenance of diabetes and cognitive decline. Moreover, there is a need for data on the role of central *vs* peripheral IGF1 levels and the subsequent impact on cognitive impairment and brain atrophy.

THE IGF1 SYSTEM

Transportation

IGF1 is a polypeptide, structurally similar to insulin, that is released in response to growth hormones secreted by the anterior pituitary[52]. While synthesized predominantly by hepatocytes in the liver and released into general circulation, both paracrine and autocrine functions contribute through local tissue synthesis of IGF1. The concentration of IGF1 is greatest during perinatal development and decreases markedly into adulthood. IGF1R are expressed in nearly all neural cells of the CNS, being most highly expressed in the cortex, hippocampus, cerebellum, brainstem, hypothalamus, and spinal cord[53].

The blood brain barrier and blood-cerebrospinal fluid barrier are the two primary routes involved with transporting systemic IGF1 into the brain. Both barriers utilize lipoprotein receptor-related proteins along with IGF1R as transporters to enter the brain[54,55]. However, the bioavailability of IGF1 is largely determined by the amount of hormone bound to IGF binding proteins (IGFBPs). Most circulating IGF is bound by IGFBPs, which are proteins that control the distribution and functional capabilities of IGF1 throughout the body. Six different IGFBPs modulate the activity of IGFs *via* binding affinities exceeding that of its respective receptor and, thus, help regulate the amount of IGF1 that enters the brain[56].

Signaling pathways

The role of IGF1 is dependent on its binding to insulin-like peptide receptors. The three most important include the IGF1R, IR, and a hybrid receptor formed from heterodimer α-β IR and IGF1R subunits[53,57]. These receptors are important to the functional efficacy of IGF1 and have defined downstream molecular pathways. As part of the tyrosine kinase receptor family, activation of IGF1R leads to the signaling of either the mitogen-activated protein kinases-extracellular signal-related kinase (MAPK-ERK) or phosphoinositide 3-kinase (PI3K)-Akt pathways[53,57]. These pathways are involved in several important cellular processes including the regulation of gene transcription, apoptosis, oxidative stress, and cellular proliferation and differentiation.

The affinity of IGF1 varies among the three receptors with the highest affinity for IGF1R. Activation of the IGF1R is capable of directly stimulating the RAS-ERK pathway, leading to the modulation of gene transcription by way of activating ETS-like transcription factor, ELK1[57]. The capacity of insulin-like peptide receptors to initiate downstream molecular activity is modified in part by the recruitment of insulin receptor substrate (IRS) scaffolding proteins[57-59]. This scaffolding helps adjust pathway choice following receptor phosphorylation. The result is activation of PI3K-Akt and subsequent expression of downstream effectors, including glycogen synthase 3 kinase (GSK3β) and mammalian target of rapamycin[53,57,60].

Relationship to the insulin system

IGF1 acts primarily through binding to the IGF1R, but also shares with insulin the capacity to bind the IR and hybrid receptor[53,56,57]. Insulin is produced exclusively by β-cells of the pancreas and, hence, is strictly transported in the systemic circulation. The amount of insulin capable of entering the brain varies considerably[54,55]. Unlike IGF1, insulin appears not to be locally synthesized in adult brain cells[53,56]. Similar to IGF1, IR located on endothelial and epithelial cell membranes allow insulin to be transported into the brain from systemic circulation. IRs are concentrated mostly in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, and cerebellum[55]. The movement of systemic insulin into the brain is not controlled by binding proteins.

Both insulin and IGF1 produced in the periphery contribute to varied physiological processes. Proper peripheral IGF1 activation is necessary for insulin secretion from the pancreas and, hence, is implicated in many facets of diabetes[61]. However, their functions differ once entering the brain. IGF1R are expressed at notably higher rates in the brain than the rate IGF1 is synthesized. This differential suggests that active transport of IGF1 into the brain is required to furnish sufficient IGF1 for proper neuronal function[47-49]. For example, peripheral IGF1 supplies the brain with

information regarding body mass, is related to neural plasticity and cognitive processes, and attenuates cognitive impairment induced by diabetes[51,62,63]. Deficiency of IGF1 can also lead to hippocampal atrophy and impaired learning[64]. Indeed, IGF1 in the brain is required for proper tissue growth in both the brain and periphery, as well as sufficient glucose regulation and insulin sensitivity[65,66].

Insulin in the periphery is well-known for its role in glucose regulation and communication with the brain to maintain energy homeostasis. Similar to IGF1, insulin is involved in modifying BBB permeability in the brain[55] with T2D patients showing greater permeability of the BBB[67]. Insulin also acts on the PI3K and MAPK signaling cascades to enhance neuronal survival, plasticity, and subsequent cognitive processes[55,68,69]. With that said, insulin does not necessarily regulate glucose activity in neuronal cells after entering the brain. Rather, insulin modulates energy homeostasis through its actions at the level of the hypothalamus[70].

INTEGRATING SEX HORMONES INTO DIABETES AND IGF1

Diabetes is associated with imbalances in sex steroid hormone levels. This is not surprising as androgens and estrogens are known to play an important role in body composition[71] while maintaining glucose and lipid homeostasis[72,73]. Research into these imbalances suggests a complex relation between estradiol (E2) and insulin insensitivity. Several studies have reported that postmenopausal women with T2D have increased levels of circulating E2[27,74,75]. Elevated E2 has been correlated with the development of insulin resistance and T2D in these women[76,77]. Nevertheless, there are at least two studies that have shown inconsistencies between E2 levels and the development of diabetes in postmenopausal women[78,79].

There is also a link between high levels of E2 and diabetes in men. Diabetic men have shown relatively high basal levels of E2[27,78], while men with higher levels of circulating E2 have an increased risk of developing T2D[80]. Although this may simply be a product of higher body fat content as adrenal androgens are readily converted to E2 in adipose tissue[81-83], two studies reported E2 results in men were independent of obesity[78,80].

Findings with animal models suggest an opposite conclusion for E2 and diabetes, at least during reproductive ages. Male mice with streptozotocin-induced insulin insensitivity are more likely to develop diabetes than their female cohorts. This increased risk of diabetes in the males can be attenuated with E2 supplements[84]. Also, mice lacking the alpha subtype of estrogen receptor (ERα) have been reported to develop insulin insensitivity[85]. In contrast, these data in animals mirror those from postmenopausal women in which glucose homeostasis was positively impacted with estrogen therapy in the short term[86].

Sex differences in androgen-diabetes relations have also been reported. Postmenopausal women with diabetes displayed elevated circulating testosterone (TS) levels[27,75]. Reports suggest that premenopausal women with higher levels of TS[76,79], as well as female mice administered the androgen[84], had a greater risk of developing diabetes. Another example is the link between T2D development and hyperandrogenism experienced by patients with polycystic ovarian syndrome[87]. Still, much like E2, there are also studies that dispute these reports, particularly in postmenopausal women[77,78].

A clear sex difference is also indicated in that diabetic men tend to have either lower total, free, or bioavailable TS than healthy men[27,88,89]. Indeed, men with the highest levels of TS were at the lowest risk and men with lowest levels of TS were at highest risk for developing T2D[78,79,90]. Moreover, men undergoing androgen deprivation treatments for prostatic cancer had a greatly increased risk of developing T2D[91]. Yet again, these reports are not without contradiction[92] and some studies found this relationship to be dependent on obesity[80,93].

Taken together, there are clear inconsistencies in the findings on sex hormones and diabetes. There is also an apparent lack of research focusing on sex hormones in premenopausal diabetic women that should be addressed[26]. It is again important to note that many studies fail to acknowledge the possible relation of sex hormones to the IGF1 system. Findings with serum E2 data are consistent with findings from meta-analyses examining IGF1[24,25]. Their proposed U-shaped association of IGF1 and T2D fits into the well-defined mechanistic relationship between E2 and IGF1, described in more detail below. The relation between sex hormones and IGF1 suggests that a delicate hormonal balance is likely an important facet of diabetes-induced brain and cognitive impairment.

NEUROPROTECTION: SEX HORMONES AND IGF1

Estrogen and IGF1

An intriguing feature of neuroactive hormones is their ability to protect the CNS from damage, especially in regards to estrogen. ER activation is implicated in the maintenance of various metabolic processes that are also associated with diabetes, including glucose homeostasis and obesity[94,95]. Only recently has research with animal models focused on neuroprotection from IGF1-E2 interactions. Evidence suggests that neuroprotective properties of E2 are directly related to receptor activities of insulin-like peptide receptors, mainly IGF1R. E2 and IGF1 work in tandem to reciprocally modulate and facilitate ER and IGFIR activation of the PI3K-Akt and MAPK-ERK signaling cascades[96-100].

IGF1 shows differential sensitivities to the two

estrogen receptor subtypes with ERα being more sensitive than ERβ[97,101]. Selective inhibition of IGF1R, for instance, downregulates ERα expression in the hypothalamus, hippocampus, and cerebral cortex, with the only significant changes of ERβ occurring in the cerebellum[38]. Many glial and neuronal cells in the brain express IGF1R and both ER subtypes[102]. In particular, ERα is uniquely capable of increasing IGF1R activity of downstream PI3K-Akt signaling in rodent models[103,104]. ERα activation also increases the binding of p85 and IRS-1 regulatory subunits of PI3K and, thus, may be one mechanism assisting in Akt pro-survival signaling through the IGF1R[39,97] (Figure 1).

Administration of E2 to mice increased IGF1R and ERα activity in the brain, enabling activation of IGF1R and downstream PI3K-Akt pathway signaling[97]. Similarly, IGF1 and insulin modulated ER effects on gene transcription and the PI3K-Akt-GSK3β signaling cascade[38,98,103,105,106]. GSK3β is a protein kinase known particularly well for its role in glycogen synthesis. However, as reviewed by Jacobs *et al*[60], recent attention has turned to the dual pro- and anti-apoptosis capabilities of GSK3β regulated through multiple different pathways. Indeed, the neuroprotective effects of IGF1 may be consequent to Akt-derived inhibition of GSK3β in a hypoxic state[107] (Figure 1).

Activation of the MAPK pathway is another important signal transduction pathway involved with regulating gene transcription and cellular proliferation and differentiation, particularly in cancer[108]. However, multiple studies have demonstrated that the neuroprotective properties of estrogen are also derived from its ability to regulate MAPK signaling in the brain[38]. Both estrogen and IGF1 can facilitate MAPK signaling through the IGF1R, with IGF1 increasing ERα activities in the presence of E2[104]. Akt inhibitors are capable of nullifying the neuroprotective effects of IGF1 and E2 regardless of MAPK signaling[99,104], while ERK suppression increases PI3K-Akt activity *via* ER and IGF1R heterodimers[39]. Thus, it appears the PI3K-Akt pro-survival signaling cascade is the most involved with the neuroprotective coupling of E2 and IGF1[39].

It is important to note that IGF1 and E2 have a remarkable reciprocity. Inhibition of ER activity can downregulate IGF1R expression in the hippocampus[109], a brain region known to atrophy in patients with diabetes and glucose intolerance[110-112]. Similarly, IGF1 has the capacity to upregulate ERα in the hippocampus and is impaired following administration of IGF1R antagonists[109]. Agonists or antagonists of either hormone can respectively facilitate or inhibit the neuroprotective and memory enhancing properties of the other[96,109,113-116]. This has led some to suggest that cooperation between IGF1R and ER is required for many E2-induced neuroprotective processes. The present section does not, however, do justice to the complexity of the relation between estrogen and IGF1 receptors. A fuller explanation can be found in one of several reviews[37-39,101,109,117].

Androgens and IGF1

Far less research has examined a functional link between IGF1 and androgens in the brain. This is an unfortunate but common trend in neuroendocrinology. Estrogens are the most intensely studied gonadal hormone, despite estrogens and androgens sharing metabolic pathways and functional properties. Much of the current literature on IGF1-androgen relations are directed at the periphery, particularly prostate cancer and motor systems, for which there are a number of recent reviews[118,119]. Few studies have examined IGF1-androgen interactions in neuroprotection[120,121] and none, to our knowledge, have empirically examined this interaction in diabetes. Therefore, we have relied on peripheral data, often from *in vitro* experiments, to extrapolate the androgen receptor (AR) brain discussion.

There is evidence that the two main androgens, TS and dihydrotestosterone (DHT), are capable of neuroprotection through binding the AR[122-126]. Similar to ERα, androgen activation of the AR in mouse vas deferens epithelial cells can modulate the p85 regulatory subunits of PI3K and subsequently trigger Akt expression (Figure 1). Inhibiting the AR prevents these signaling effects[127]. Phosphorylation of MAPK and Akt can also increase AR activation in low androgen and estrogen concentrations, as well as increase the neuroprotective activities of ERα and AR[128]. Recent findings showed that DHT, which has a higher affinity than TS for the AR, prevents apoptosis in a C6 glial cell line through the PI3K-Akt signaling cascade[129]. These effects were also impaired by inhibition of PI3K and suggest a functional relationship between apoptosis and AR activities.

Interestingly, studies have demonstrated that binding of DHT to the transmembrane AR impairs MAPK and PI3K signaling and subsequent neuroprotection from DHT or E2[130-132]. This suggests that nuclear activation of the AR by DHT is likely one mechanism behind DHT's neuroprotective properties[130]. DHT may also interact with effectors downstream of ER and IGF1R signaling. Both TS and DHT can activate the MAPK-ERK signaling cascade[132] which has been shown to induce ribosomal S6 kinase (Rsk) expression. Rsk signaling can lead to the inhibition of the pro-apoptosis Bad protein and the activation of downstream effectors including the ER, GSK3β and ELK1[133] (Figure 1).

One possible explanation for the neuroprotective role of androgens is the conversion in the steroid metabolic cascade of TS into E2 by the enzyme aromatase. That is, TS may be involved in neuroprotection only to the extent that TS is a precursor for E2, which is capable of activating MAPK or PI3K signaling through the ER and IGF1R. The aromatization of TS into E2, as well as the aromatase enzyme, have been suggested to play an important role in neuroprotection[134-139].

The ratio of endogenous TS to E2, and subsequent influences of aromatized TS, is indeed a topic of recent interest[26]. Increased local synthesis of E2 from elevated aromatase expression is seen in models

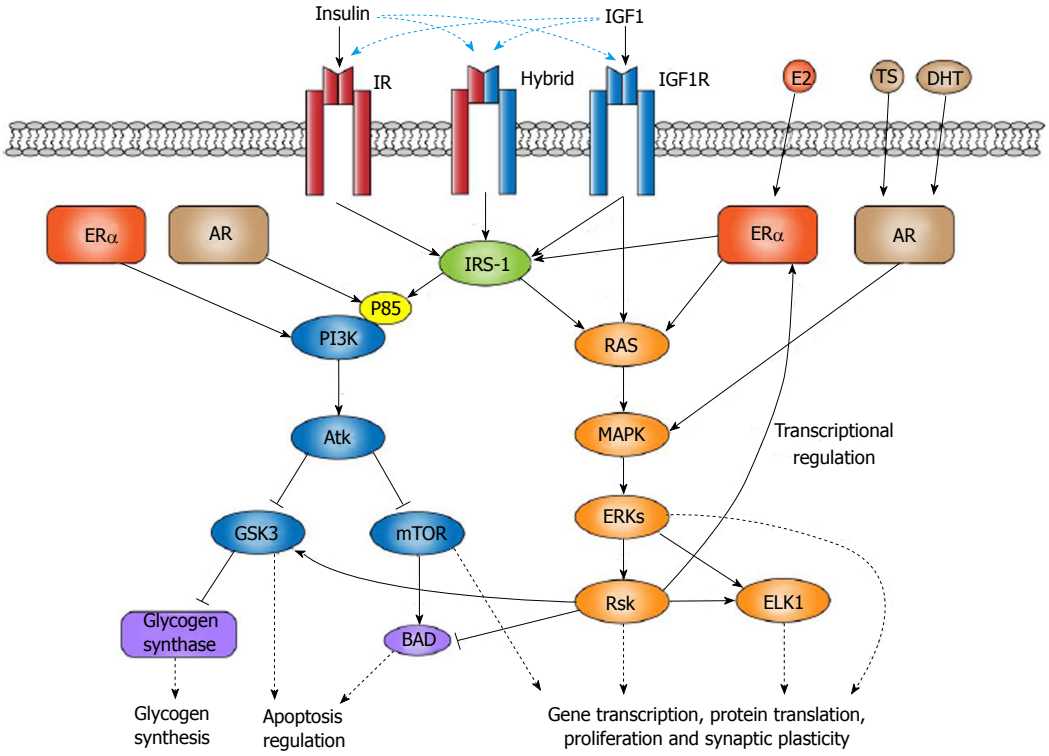

Figure 1 Similar signaling cascades involved with neuroprotection for insulin-like peptides and sex hormones. The insulin receptor (IR), insulin-like growth factor 1 receptor (IGF1R), and insulin-IGF1 hybrid receptor enact their neuroprotection through the mitogen-activated protein kinases-extracellular signal-related kinase (MAPK-ERK) or phosphoinositide 3-kinase (PI3K)-Akt pathways signaling cascades. Although IGF1R can directly activate the RAS-ERK pathway, both the insulin-like peptide receptors and the estrogen receptor alpha (ERα) firstly interact with insulin receptor substrate 1 (IRS-1) scaffolding proteins. ERα and the androgen receptor (AR) can also directly modulate PI3K-Akt and MAPK-ERK signaling. Both IRS-1 and p85 binding of PI3K are increased with ERα activation, leading to downstream Akt-derived inhibition of glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin (mTOR). GSK3, specifically, is involved with glycogen synthesis, while both effectors are involved in apoptosis. A similar effect may occur with AR's ability to modulate p85 binding to PI3K. AR-induced MAPK-ERK signaling also results in ribosomal S6 kinase (Rsk) expression that can inhibit the pro-apoptosis bcl-2-associated death promoter protein, as well as effects on the ER, GSK3, and the ETS-like transcription factor, ELK1. Solid black arrows indicate downstream interaction. Dashed black arrows represent the influence of kinases or proteins on the cellular environment. Dashed blue arrows represent the binding capabilities of IGF1 and insulin across all three receptor types.

of neuroprotection from other brain disorders, *e.g.*, stroke[140]. More pertinent to this review, streptozotocin-induced diabetes causes a considerable reduction in aromatase synthesis in female and male reproductive systems[141]. Notably, inhibition of aromatase decreases E2 and impairs insulin sensitivity and peripheral glucose disposal in healthy males[142], although the influence this may have on brain integrity and cognitive outcomes remains debated[143].

Another explanation places greater emphasis on the other pathway in the steroid metabolic cascade leading to DHT. Metabolites of DHT, 3α-Diol and 3β-Diol, are also bioactive and may bind the ER or insulin-like peptide receptors to initiate MAPK or PI3K signaling cascades. Indeed, research shows that 3α-Diol stimulated PI3K-Akt signaling enhances cell survival in the prostate[144]. Similarly, DHT metabolites may influence transcriptional activities of nuclear ER by modulating ER-induced MAPK or PI3K signaling cascades.

Few *in vivo* studies examining these sex steroid metabolites have focused on MAPK or PI3K signal cascades in the brain. There is, however, evidence that 3α-Diol inhibits protein kinase A expression in the rat hippocampus[145]. Others have reported that streptozotocin-induced diabetic mice had lower levels of TS and 3α-Diol in the cerebral cortex, and lower levels of DHT and 3α-Diol in the spinal cord[146]. It is still unclear, though, whether 3α-Diol and 3β-Diol interact with or initiate the MAPK or PI3K signaling cascades following activation of the ER, AR, or, possibly, IGF1R.

None of these explanations clarify fully the ability of the AR to directly trigger these signaling cascades. We do not aim to discount the neuroprotective mechanisms of ER and AR, or the clear link between E2 and IGF1 processes in neuroprotection. Rather, we simply suggest that androgen-derived neuroprotection may be intertwined with IGF1, the activation of insulin-like peptide receptors, and/or the IGF1R and ER coupling. Given the common signaling pathways between these hormones, we suggest future research should aim to include androgens and AR activities into the ER-IGF1R neuroprotective coupling, as well as serum comparisons in brain-health outcomes of diabetic patients.

CONCLUSION

The reciprocity of IGF1 and estrogen in neuroprotective processes is well-established in cell cultures and

animal models[38]. Interactions between androgens and IGF1 may also play an important role in the E2-IGF1 neuroprotective coupling. Both estrogens and androgens enact their neuroprotection through similar, but not identical, signal transduction pathways. Recognition of this has led us to consider the possibility that these sex hormones may work together with IGF1 and insulin-like peptide receptors to modulate MAPK and PI3K signaling and their neuroprotective properties.

Regulation of MAPK and PI3K activity may also be a driving force behind the structural changes, atrophy of brain regions, or functional changes, often observed in diabetic patients. Drawing conclusions from imaging data in humans to those found in animal models is indeed difficult. Nevertheless, there is a need for a clearer mechanistic explanation grounding the cognitive decline and brain abnormalities observed in diabetic patients.

Future studies in human research on diabetic brain integrity should integrate hormone titer measures to help substantiate sex differences in brain-health outcomes of diabetic patients. This approach may also assist in identifying region-specific brain abnormalities resulting from fluctuations in IGF1 and sex hormones between men and women. Moreover, animal models examining the E2-IGF1 coupling in neuroprotection should employ streptozotocin-induced diabetes, as well as the possible role of androgens and AR activities. These conclusions warrant further examination of the variability present in cognitive and brain-health outcomes for patients with diabetes as a result of sex hormone relations to IGF1, insulin, and the insulin-like peptide receptors.

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ORIGINAL ARTICLE

Basic Study

High fat diet dysregulates microRNA-17-5p and triggers retinal inflammation: Role of endoplasmic-reticulum-stress

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Abstract

AIM

To elucidate how high diet-induced endoplasmic reticulum-stress upregulates thioredoxin interacting protein expression in Müller cells leading to retinal inflammation.

METHODS

Male C57Bl/J mice were fed either normal diet or 60% high fat diet for 4-8 wk. During the 4 wk study, mice received phenyl-butyric acid (PBA); endoplasmic reticulum-stress inhibitor; for 2 wk. Insulin resistance was assessed by oral glucose tolerance. Effects of palmitate-bovine serum albumin (BSA) (400 μmol/L) were examined in retinal Müller glial cell line and primary Müller cells isolated from wild type and thioredoxin interacting protein knock-out mice. Expression of thioredoxin interacting protein, endoplasmic reticulum-stress markers, miR-17-5p mRNA, as well as nucleotide-binding oligomerization domain-like receptor protein (NLRP3) and IL1β protein was determined.

RESULTS

High fat diet for 8 wk induced obesity and insulin resistance evident by increases in body weight and impaired glucose tolerance. By performing quantitative real-time polymerase chain reaction, we found that high fat diet triggered the expression of retinal endoplasmic reticulum-stress markers ($P < 0.05$). These effects were associated with increased thioredoxin interacting protein and decreased miR-17-5p expression, which

were restored by inhibiting endoplasmic reticulum-stress with PBA ($P < 0.05$). *In vitro*, palmitate-BSA triggered endoplasmic reticulum-stress markers, which was accompanied with reduced miR-17-5p and induced thioredoxin interacting protein mRNA in retinal Müller glial cell line ($P < 0.05$). Palmitate upregulated NLRP3 and IL1β expression in primary Müller cells isolated from wild type. However, using primary Müller cells isolated from thioredoxin interacting protein knock-out mice abolished palmitate-mediated increase in NLRP3 and IL1β.

CONCLUSION

Our work suggests that targeting endoplasmic reticulum-stress or thioredoxin interacting protein are potential therapeutic strategies for early intervention of obesity-induced retinal inflammation.

Key words: High fat diet; Palmitate; Endoplasmic-reticulum-stress; Inflammation; Thioredoxin-interacting protein; Micro-RNA 17-5p

Core tip: We previously showed that high fat diets (HFD) induced retinal inflammation and vascular dysfunction. These results were associated with an increase in thioredoxin interacting protein (TXNIP) at the mRNA and protein level. Here, we examined the mechanisms by which HFD triggers retinal TXNIP. Interestingly, we found that HFD/palmitate triggers ER-stress mediators including the inositol requiring enzyme 1, an RNAse that can degrade number of mRNAs including the microRNA; miR-17-5p and sustains TXNIP expression. Inhibiting ER-stress prevented the increase in TXNIP *in vivo* and in Müller cells, the main glia in the retina. Deletion of TXNIP blunted NLRP-3 inflammasome and IL-1β release in Müller cells.

Coucha M, Mohamed IN, Elshaer SL, Mbata O, Bartasis ML, El-Remessy AB. High fat diet dysregulates microRNA-17-5p and triggers retinal inflammation: Role of endoplasmic-reticulum-stress. *World J Diabetes* 2017; 8(2): 56-65 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i2/56.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i2.56

INTRODUCTION

Obesity, recently upgraded from a mere risk factor to a disease state, is affecting one third of United States population[1]. Clinical evidence showed that obesity not only can accelerate developing type-2 diabetes and cardiovascular complications, but also induce retinal microvascular abnormalities, which eventually leads to visual impairments[2,3]. High fat diets (HFD) together with the improper physical activity are the culprit in the obesity-induced pre-diabetes. Therefore, there is an urgent need to unravel the mechanisms involved in HFD-mediated neurovascular abnormalities. Our lab has previously shown that consumption of high caloric diet saturated fatty acids induced retinal inflammation and microvascular dysfunction *via* upregulating the expression of thioredoxin interacting protein (TXNIP); a regulator of the antioxidant thioredoxin; and activating NOD (NOD)-like receptor protein (NLRP3)-inflammasome[4]. Similar observations showed the contribution of TXNIP/NLRP3-inflammasome signaling pathway to the development of various disorders in other organs[5-7]. However, molecular mechanisms by which HFD triggers early TXNIP expression in the retina are still unclear.

MicroRNAs are small non-coding RNAs that control the translation and transcription of various genes *via* annealing to the complementary sequences in the 3′ untranslated region of their target gene[8]. To date, several miR classes have been identified to be involved in development of obesity, diabetes and diabetic complications[9]. Bioinformatic analysis of the TXNIP 3′ UTR identified 11 possible miRNAs that can regulate its expression including miR-130/301, miR-128, miR-148/152, miR-135, miR-106/302, miR-17-5p/20/93.mr/106/519.d, miR-128, miR-15/16/195/424/497, miR-106/302, miR-148/152. Nevertheless, levels of miR-17-5p have been reported to rapidly decline under stress condition resulting in enhancing TXNIP expression[10,11].

Unfolded protein response (UPR) is an adaptive response, which prevents the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER). The UPR is transduced by three major ER-resident stress sensors, namely Protein Kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1). However, when protein misfolding exceeds the capacity of the UPR an ER-stress will result that triggers programmed cell death. So far, ER-stress has been shown to play a critical role in the pathogenic progression of various chronic diseases including diabetic retinopathy (reviewed in[12-14]). Among UPR pathways, IRE1α, an ER bifunctional kinase/RNase has been shown to destabilize number of RNA and microRNA including miR-17-5p in pancreatic beta-cells[10,11]. Several studies reported the impact of HFD and its related metabolite such as free fatty acid in inducing ER-stress[15-17]. In the current study we were trying to decipher the underlying mechanisms that link HFD-mediated ER-stress to retinal inflammation. Here, we tested the hypothesis that HFD-mediated ER-stress upregulates TXNIP mRNA expression *via* dysregulating miR-17-5p resulting in retinal inflammation.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in agreement with Association for Research in Vision and Ophthalmology

Table 1 The sequence of the polymerase chain reaction primers used in the experiments

Gene	Forward	Reverse
18 S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC
XBP1	ACACGCTTGGGAATGGACAC	CCATGGGAAGATGTTCTGGG
XBP1-SPLICED	GAGTCCGCAGCAGGTG	GTGTCAGAGTCCATGGGA
PERK	AGTCCCTGCTCGAATCTTCCT	TCCCAAGGCAGAACAGATATACC
IRE1α	GGGTTGCTGTCGTGCCTCGAG	TGGGGGCCTTCCAGCAAAGGA
ATF6	TGCCTTGGGAGTCAGACCTAT	GCTGAGTTGAAGAACACGAGTC
CHOP	CTGGAAGCCTGGTATGAGGAT	CAGGGTCAAGAGTAGTGAAGGT
TXNIP	AAGCTGTCCTCAGTCAGAGGCAAT	ATGACTTTCTTGGAGCCAGGGACA

statement for use of animals in ophthalmic and vision research, and Charlie Norwood VA Medical Center Animal Care and Use Committee (ACORP#15-04-080). 6-8 wk old male C57BL6/J mice (Stock 000664, Jackson Laboratory, ME, United States) were used in the *in vivo* studies. For the long term study, mice were fed ad libitum with normal rat chow (7% fat) or HFD [36 g %, 251 kJ (60 kcal) %fat] (F2685 Bioserv, Frenchtown, NJ, United States) for 8 wk. For the short term study, mice were fed either normal diet (ND) or 60% HFD for 2 wk. Mice were then kept on HFD for additional 2 wk while receiving an ER-stress inhibitor [Phenyl-butyric acid (PBA), 100 mg/kg] or vehicle. PBA was dissolved in DMSO/PBS and administered *via* oral gavage 5 d/wk. Mice were weighed weekly to track the increase in the body weight.

Intra-peritoneal glucose tolerance test

Mice went overnight fasting, and their fasting plasma blood glucose was measured as the baseline. Then all mice received an intraperitoneal injection of glucose (2 g/kg). Blood glucose levels were measured at different time points till 120 min after the glucose injection using a glucometer.

In-vitro studies

The rat retinal Müller glial cell line (rMC-1) was obtained originally from V. Sarthy (Department of Ophthalmology, Northwestern University, Chicago, IL, United States)[18]. Primary mouse Müller Cells from WT and TKO mice were isolated and cultured as described previously[19]. Cells were grown to confluency in complete media (DMEM, 10% vol/vol. FBS, 1% vol/vol. penicillin/streptomycin). Sodium palmitate (Cat.# P9767; Sigma-Aldrich, St. Louis,MO, United States) was dissolved in 50% ethyl alcohol, then added drop-wise to preheated 10% endotoxin- and fatty acid-free BSA (Cat.# 22070017; Bioworld, Dublin, OH) in DMEM at 50 ℃ to create an intermediate stock solution of palmitate coupled to BSA (Pal-BSA). Confluent cells were switched to serum-free medium for overnight then were treated for 6 h with Pal-BSA solutions (400 μmol/L final concentration). Equal volumes of 50% ethyl alcohol with BSA alone served as control. In another set of rMC-1, cells were serum starved for 4 h then treated with PBA (1 mmol/L, Cat.#P21005, Sigma-Aldrich) or IRE1α inhibitor (STF-083010, 50 μmol/L) for 2 h then palmitate was added and kept overnight.

Quantitative real-time PCR

A one-step quantitative RT-PCR kit (Invitrogen) was used to amplify 10 ng retinal mRNA as described previously[4]. PCR primers (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA, United States). Quantitative PCR was conducted using StepOnePlus qPCR system (Applied BioSystems, Life Technologies). The percent expression of various genes was normalized to 18S.

Micro-RNA detection

MirVana PARIS kit (Cat.# AM1556, Invitrogen) was used for miRNA isolation according to manufacturer's protocol. Reverse transcriptase reactions; including samples and no-template controls; were run using TaqMan® MicroRNA Reverse Transcription Kit (Cat.# 4366596, Applied Biosystems) as described previously[20]. PCR amplification was performed using TaqMan® Universal PCR Master Mix (Cat.# 4324018, Applied Biosystems) according to manufacturer's protocol. The percent expression of miR-17-5p was normalized to U6.

Western blot analysis

Retinas were isolated and homogenized in cell disruption buffer as described previously[21]. Müller cells were harvested by scraping thoroughly with cell scraper after the addition of cell disruption buffer. Samples (25 μg protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with the primary antibodies; anti-TXNIP (Cat.# K0205-3 MBL Abacus ALS Australia and Cat.# 403700, Invitrogen, Grand Island, NY), anti-NLRP-3 (Cat.# LS-B4321, LifeSpan Biosciences, Inc, Seatle, WA), anti-IL1β (Cat.# ab9722, Abcam, Cambridge, MA, United States) then reprobed with housekeeping gene; anti-GAPDH (Cat.# 5174, Cell Signaling, Danvers, MA, United States), anti-tubulin (Cat.# ab4074, Abcam, Cambridge, MA, United States) or anti-actin (Cat.# a5060, Sigma-Aldrich) to confirm equal loading. The primary antibody was detected using a horseradish peroxidase (HRP) and enhanced chemiluminescence. The films were scanned and the band intensity was quantified using densitometry software version 6.0.0 Software from alphaEaseFC (Santa Clara, CA) and expressed as relative

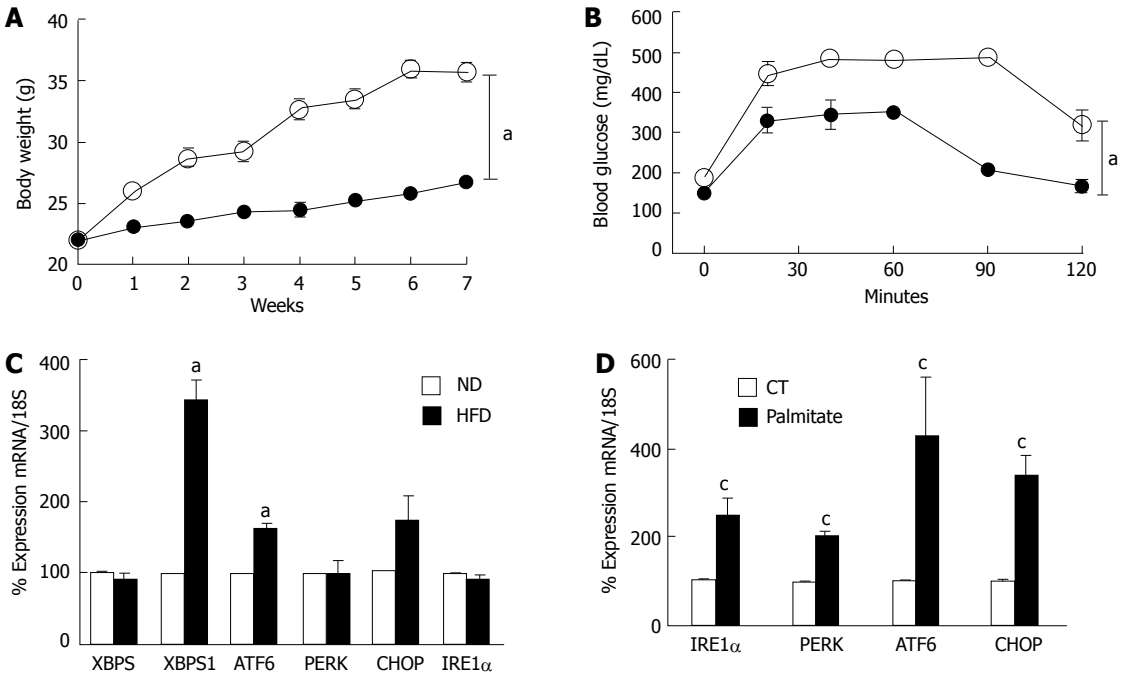

Figure 1 High fat diet/palmitate triggered endoplasmic-reticulum-stress markers in retina and Müller cells. A: Total body weight (grams) recorded weekly was significantly higher in mice fed with HFD for 8 wk compared to ND; B: Glucose tolerance was impaired after 8 wk of HFD compared to ND; C: Realtime PCR showing increases in mRNA levels of XBP1S and ATF6, while no change in XBP1, PERK, CHOP and IRE1α mRNA in retina after 8 wk of HFD compared to ND; D: Realtime PCR showing significant increases in IRE1α, PERK, ATF6 and CHOP mRNA levels in rMC1 treated with palmitate compared to control (CT) ($^{a}P < 0.05$ *vs* ND, $n = 3$-4 and $^{c}P < 0.05$ *vs* CT, area under the curve across all the time points was calculated, $n = 3$-4). HFD: High fat diet; PERK: Protein Kinase RNA-like endoplasmic-reticulum kinase; XBP: Xbox binding protein; ATF6: Activating transcription factor 6; CHOP: CCAAT-enhancer-binding protein homologous protein; IRE1: Inositol requiring enzyme 1.

optical density (OD).

Statistical analysis

All the data are expressed as mean ± SD or SEM. Differences between ND *vs* HFD and control *vs* palmitate were tested using two-sample *t* tests. One-way ANOVA followed by Bonferroni post-hoc multiple comparisons to assess significant differences between 3 or more groups (Graphpad-Ver.6). For body weight and blood glucose measurements, area under the curve (AUC) across all the time points was calculated. A series of 2 gene (WT *vs* KO) × 2 treatment (TRT) (no *vs* yes) ANOVAs with interaction were used to determine the effect of palmitate on NLRP3 and IL1β. A Bonferroni post-hoc multiple comparison test was used for significant interactions. Significance for all tests was determined at alpha = 0.05.

RESULTS

HFD/palmitate triggered ER-stress markers in retina and Müller cells

Several studies showed that HFD or palmitate triggers ER-stress in different organs and cell types[17,22-24]. Therefore, we checked the levels of various ER-stress markers in the retina isolated from mice fed with HFD, and rMC1 treated with palmitate. HFD for 8 wk induced obesity and impaired glucose tolerance indicated by an increase in body weights (Figure 1A) and glucose levels (Figure 1B) across the different time points compared to ND. We also found that HFD induced an increase in XBPS1 and ATF6 mRNA levels only, while, there was no change in XBP1, PERK, CHOP and IRE1α (Figure 1C). In order to study the role of Müller cells in HFD-induced inflammation, rMC-1 were treated with 400 μmol/L palmitate coupled to bovine serum albumin (Pal-BSA) for 6hr. Palmitate; a saturated fatty acid that is increased in plasma following a HFD[25]; significantly upregulated IRE1α, PERK, ATF6 and CHOP (Figure 1D).

HFD/palmitate induced TXNIP upregulation and miR-17-5p dysregulation in retina and Müller cells

Our lab has previously reported that HFD and palmitate can induce TXNIP mRNA expression in whole retina and retina endothelial cells respectively[4]. However, the upstream events by which HFD/palmitate trigger TXNIP expression are still unclear. In agreement with the previous study, we found that 8 wk of HFD and palmitate led to an upregulation of TXNIP mRNA levels in whole retina and Müller cells (Figure 2). These results were associated with miR-17-5p dysregulation in both whole retina and Müller cells (Figure 2).

PBA mitigated HFD-mediated ER-stress

To verify the role of ER-stress in HFD-induced TXNIP upregulation, mice were fed either ND or HFD for 2 wk. Then mice were kept on HFD for additional 2 wk while receiving PBA; an ER-stress inhibitor. Body weights were not changed by the HFD or PBA treatment (Figure 3A). However, blood glucose tolerance was significantly less in mice fed with HFD compared to ND after intra-peritoneal glucose tolerance test (Figure 3B). HFD-induced insulin

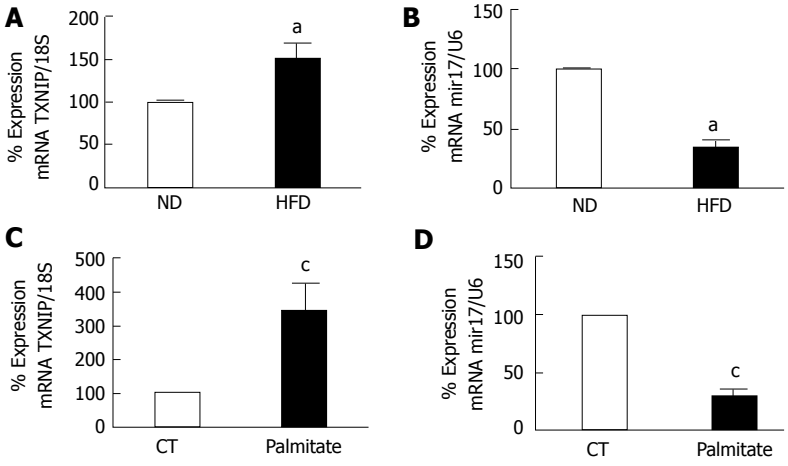

Figure 2 Realtime polymerase chain reaction. It shows significant (A) increase in TXNIP mRNA and (B) miR-17-5p dysregulation in retina after 8 wk of HFD compared to ND. Realtime PCR showing significant (C) increase in TXNIP mRNA levels (D) reduction in miR-17-5p in rMC1 treated with palmitate compared to control (CT) ($^{a}P < 0.05$ *vs* ND, $n = 3$-4 and $^{c}P < 0.05$ *vs* CT, $n = 3$). ND: Normal diet; HFD: High fat diet.

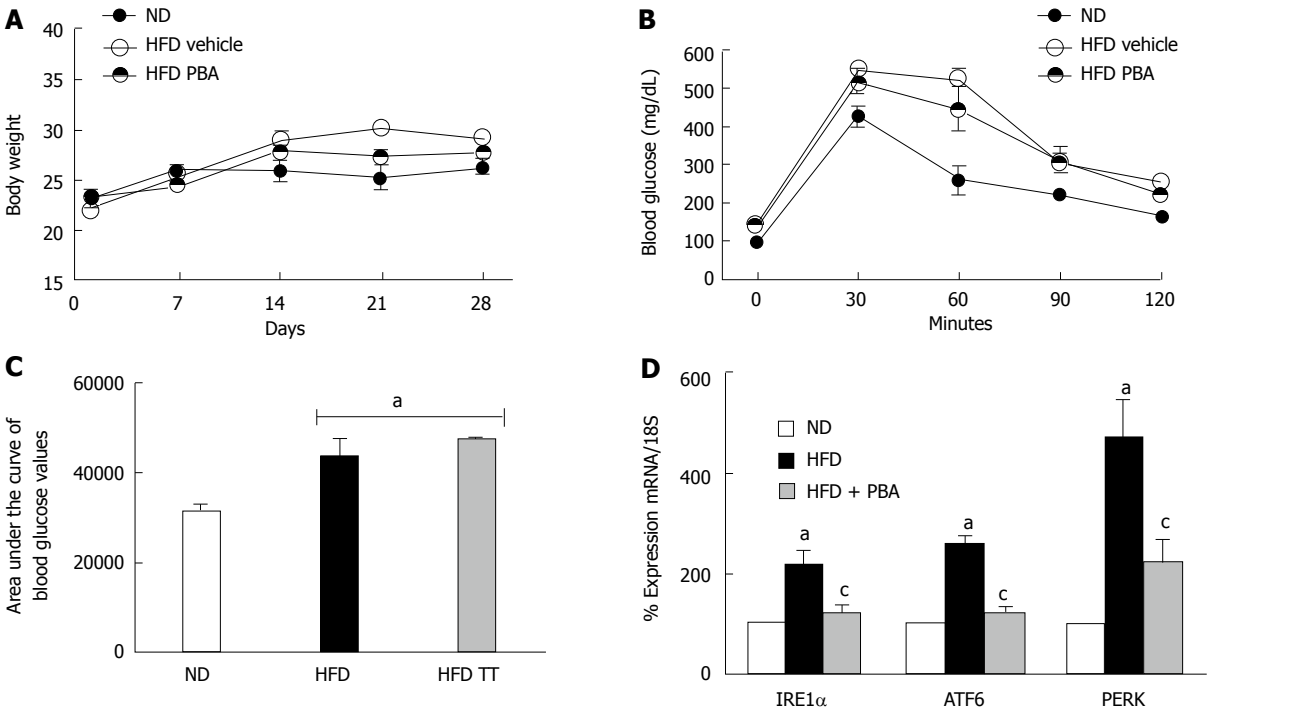

Figure 3 PBA mitigated high fat diet-mediated endoplasmic-reticulum-stress. A: Total body weight (g) recorded weekly for 4 wk was not changed among the different groups; B: Glucose tolerance was impaired after 4 wk of HFD compared to ND, and was not restored with PBA treatment; C: Statistical analysis of area under the curve showing an increase in blood glucose levels in HFD compared to ND, which was not reversed by the treatment; D: Realtime PCR showing significant increases in IRE1α, ATF6, and PERK mRNA levels in mice kept on HFD for 4 wk compared to ND, which were nullified with PBA treatment ($^{a}P < 0.05$ *vs* ND, $^{c}P < 0.05$ *vs* HFD, $n = 3$-4). ND: Normal diet; HFD: High fat diet; PBA: Phenyl-butyric acid; PERK: Protein Kinase RNA-like endoplasmic-reticulum kinase; IRE: Inositol requiring enzyme.

resistance suggested by marked increase in the area under the curve remained unaffected by inhibiting ER-stress with PBA (Figure 3C). HFD for 4 wk induced expression of retinal ER-stress markers mRNA including the RNAse IRE1α, ATF6 and PERK which were restored by PBA treatment to control level (Figure 3D).

ER-stress inhibition prevented HFD-induced TXNIP upregulation and miR-17-5p dysregulation

To establish a causal relationship of the role of ER-stress miR-17-5p and TXNIP expression, we assessed their expression in animals that were treated with ER-stress inhibitor PBA. As shown in Figure 4A, intervention with PBA treatment in HFD partially but significantly increased retinal miR-17-5p compared to untreated HFD. HFD triggered TXNIP mRNA and protein expression compared to ND, which were significantly inhibited in HFD-animals treated with PBA (Figure 4B-D). To establish a causal relationship of the role of ER-stress and activation of IRE1α in palmitate-induced TXNIP expression, rMC1 were treated for 2 h with PBA or IRE1α inhibitor prior to the addition of palmitate. As shown in Figure 4, inhibiting ER-stress or IRE1α markedly reduced the increase in TXNIP protein expression in palmitate-treated cells.

Knocking out TXNIP abolished palmitate induced inflammation in Müller cells

We recently showed that HFD induced expression

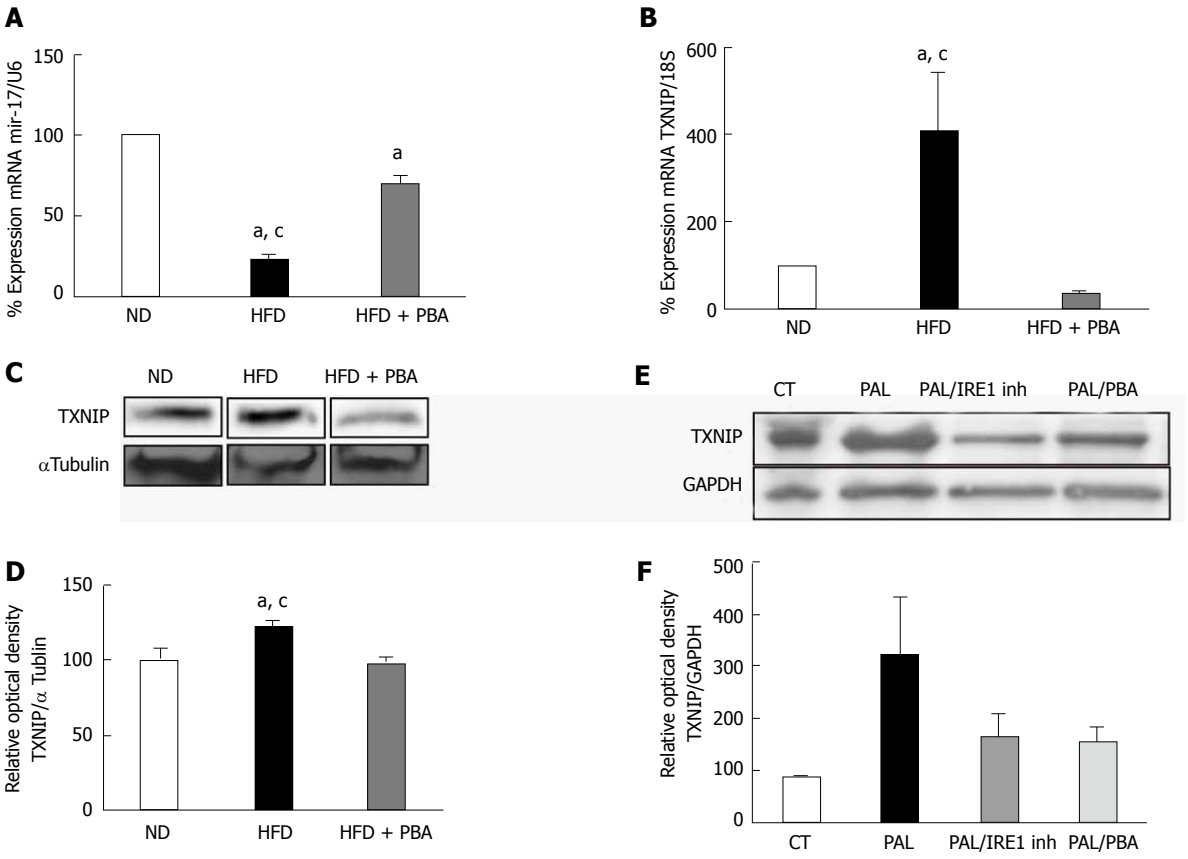

Figure 4 Dysregulation of realtime polymerase chain reaction. It shows significant (A) reduction in miR-17-5p and (B) increase in TXNIP mRNA levels in mice kept on HFD for 4 wk compared to ND, which were reversed with PBA treatment (n = 3-4); C: Representative western blot were cut from the same membrane for TXNIP and αtubulin from retina (D) Statistical analysis showed an upregulation in TXNIP expression in HFD mice compared to ND, PBA treatment nullified this effect (n = 4-5) ($^{a}P < 0.05$ *vs* ND, $^{\circ}P < 0.05$ *vs* HFD + PBA) (E) Representative western blot of TXNIP and GAPDH from rMC1 treated with palmitate (pal), after the addition of IRE inhibitor or PBA; D: Statistical analysis showed a trend increase in TXNIP expression, which is reversed by IRE inhibitor or PBA ($P = 0.076$, n = 3). HFD: High fat diet; ND: Normal diet; PBA: Phenyl-butyric acid; TXNIP: Thioredoxin-interacting protein; CT: Control; PAL: Palmitate; PAL/IRE1: Palmitate + inositol requiring enzyme 1; PAL/PBA: Palmitate + phenyl-butyric acid.

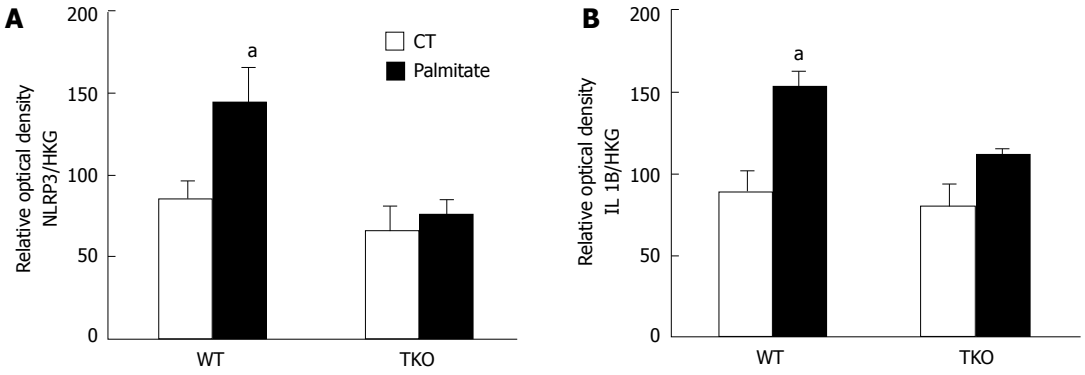

Figure 5 Statistical analysis showed an increase in. (A) NLRP3 and (B) IL1β expression following palmitate treatment in primary Müller cells isolated from WT but no effect on TKO. Actin and α-tubulin were used as housekeeping genes (HKG), to which NLRP3 and IL1β expression was normalized ($^{a}P < 0.05$ *vs* ND, n = 3).

of TXNIP in Müller cells, which was associated with increased TXNIP-NLRP3 inflammasome interaction as well as the expression of cleaved caspase-1 and IL-1β[4]. Therefore, to dissect the role of TXNIP in palmitate-mediated inflammation in Müller cells, primary Müller cells from both WT and TKO mice were used. Primary Müller cells were serum starved overnight then treated with 400 μmol/L palmitate coupled to bovine serum albumin (Pal-BSA) for 6 h. We found that palmitate led to an increase in NLRP3 and IL1β protein expression in cells isolated from WT but has no effect on cells isolated from TKO mice (Figure 5).

DISCUSSION

Central obesity and insulin resistance are hallmarks

of metabolic syndrome that comprises dyslipidemia, hypertriglyceridemia, hyperinsulinemia, hypertension, and reduced HDL cholesterol. Changes in lipid profile and accumulation of free fatty acids are highly significant in all forms of diabetes pointing to its possible link with inflammation and vascular complications (reviewed in[26]). Several studies showed the role of free fatty acids mainly palmitate in inducing pro-inflammatory response[27,28]. It should be noted that thorough understanding of the interaction between vascular and non-vascular cells is crucial for the management of retinal dysfunction. Müller cells are the principal glial cell found in the retina, which span the entire retinal layers and considered as resident innate immune cells (reviewed in[29]). Because of their unique morphology, Müller cells are considered a signaling hub that senses minute changes in retinal milieu, connecting retinal neuronal with retinal endothelial cells. In the current study we were interested in unraveling the mechanisms through which HFD leads to retinal inflammation. We also highlighted the critical role of Müller cells after the insult with the free fatty acid palmitate, which hasn't been reported so far. The main findings of this study are that (1) HFD or palmitate induced ER-stress dysregulates miR-17-5p in retina and Müller cells; (2) ER-stress triggers TXNIP expression in retina and Müller cells and (3) amplified TXNIP levels activate NLRP3, which contributes to inflammation.

Müller cells are considered major sources of inflammatory mediators, which become activated in response to various insults[19,30-32]. We and others have shown the increase of TXNIP expression in glial Müller cells due to chronic hyperglycemia[33-35] or HFD[4]. TXNIP is a physiological inhibitor of the thioredoxin system, which is one of the main antioxidant defense mechanisms in our body. TXNIP acts *via* binding to thioredoxin, making it unable to bind with other proteins (reviewed in[36]). In addition to the ability of TXNIP in inducing inflammatory cytokines *via* activating nuclear factor κB, it can act as a direct activator of NOD-like receptor protein (NLRP3)[34,37]. NLRP3-inflammasome is a component of the innate immune system responsible for initiating obesity-induced inflammation[38]. TXNIP-NLRP3 interaction results in NLRP3 complex assembly and auto-activation of caspase-1, which eventually processes pro-IL1β into its mature form leading to inflammation[38,39]. Recent studies showed that HFD and palmitate trigger ER-stress in various organs and cell types[17,22-24]. However, the link between HFD/palmitate-induced ER-stress and TXNIP expression in Müller cells is yet to be determined. Here, we observed significant activation of the unfolded protein response ER-stress chaperons in retinas from 8-wk HFD mice (Figure 1). We also observed no difference in mRNA level of IRE1α an ER-stress marker and a bifunctional kinase/Rnase in HFD. However, there was an increase in the splicing of XBP1; IRE1α downstream target; evident by 3.5-fold increase in spliced XBP-1 in HFD compared to ND, which suggests IRE1α activation. Interestingly, treatment of Müller cells with palmitate; one of the most abundant saturated fatty acids in plasma that is significantly increased following HFD[25]; led to an increase in all ER-stress markers at the mRNA level including IRE1α (Figure 1). Among UPR pathways, IRE1α has been shown to degrade key cell regulators such as the neuronal cue, netrin in the retina[39,40] and miR-17-5p in pancreatic beta-cells[10,11]. MiR-17-5p is a small noncoding RNAs that binds predominantly to the 3′-UTR of TXNIP leading to down-regulation of its expression[10]. Indeed, HFD and palmitate resulted in a significant decrease in miR-17-5p in the total retina and Müller cells, respectively, an effect that coincided with TXNIP upregulation (Figure 2). These findings support the link between HFD, ER-stress and TXNIP upregulation in Müller cells.

Epidemiological studies showed a significant reduction in miR-17-5p in omental fat and blood from obese non-diabetic subjects compared to lean subjects[41,42]. In the current study, we showed that HFD or palmitate dysregulated miR-17-5p in retina and Müller cells (Figure 2). Interestingly, retinal miR-17-5p expression is not affected by hyperglycemia or diabetes compared to normal glycemic controls (data not shown). In agreement, Lerner *et al*[10] reported similar insensitivity of miR-17-5p to high glucose treatment in pancreatic beta cells. These findings shed light on the selective sensitivity of miR-17-5p to degrade in response to HFD and palmitate. Taken together, our findings suggest that HFD-induced ER-stress uniquely triggers TXNIP expression *via* dysregulating miR-17-5p.

To dissect the role of ER-stress in regulating TXNIP expression, PBA was added to cultured rMC1 prior to palmitate treatment. PBA is an FDA approved drug for the clinical management of urea cycle disorder. PBA is a chemical chaperone that stabilizes protein conformation and in turns ER-folding (reviewed in[43,44]). Indeed, treating the cells with PBA a general ER-stress inhibitor showed a trend decrease in TXNIP expression. Similar findings were obtained by the use of a selective IRE1α inhibitor (Figure 4). However, the observed reduction didn't reach significance, which could be due to the small sample size. We overcame this limitation, by treating mice kept on HFD with PBA for 2 wk. We showed that inhibiting ER-stress significantly blunted the increase in TXNIP observed in HFD group (Figure 4), without altering insulin resistance (Figure 3). Next step we tried to verify the role of TXNIP in inflammatory response in Müller cells. Building on our previous findings that silencing TXNIP reversed palmitate-induced IL1β release and eventually cell death in endothelial cells[4], we isolated primary Müller cells from WT and TKO mice then exposed them to palmitate. We demonstrated that palmitate led to an increase in NLRP3 and IL1β expression in WT and has no effect on TKO (Figure 5), which indicates that TXNIP is responsible for inflammation in Müller cells. These results lend further support to prior findings that manifest the critical role of IL1β in mediating vascular injury in the pathogenesis of diabetic retinopathy. Kowluru *et al*[45] showed that injecting IL-1β

into the vitreous of normal rats increased cell apoptosis similar to what is observed in diabetes. Deletion of IL1β receptor prevented autocrine loop of inflammation[46] and protected retinas from diabetes-induced development of acellular capillaries[47].

In summary, clinical and experimental studies have repeatedly reported the contribution of inflammation to the pathogenesis of diabetic retinopathy (reviewed in[48,49]). Similarly, suppression of inflammation has shown protective effects *via* decreasing leukostasis, blood-retinal barrier breakdown and the acellular capillaries formation[50]. Here, we provide preliminary evidence that exposure to high fat diet and palmitate trigger retinal ER-stress and glial TXNIP expression and render the retina vulnerable to inflammation. Early intervention of ER-stress or TXNIP presents potential therapeutic strategy in obesity-induced inflammation in diabetic retinopathy.

COMMENTS

Background

The authors have previously shown that high fat diet (HFD) induced retinal inflammation and vascular dysfunction. These results were associated with an increase in the thioredoxin interacting protein (TXNIP) at the mRNA and protein level. Here, they examined the mechanisms by which HFD triggers retinal TXNIP and regulates inflammation.

Research frontiers

Currently, there is a great interest to understand how microRNA, the endogenous regulators of transcription can contribute to metabolic disorders. Here, they examined the impact of HFD or the free fatty acid palmitate on microRNA; miR-17-5p as it has been shown to regulate TXNIP mRNA expression. This study demonstrates the effect of HFD-induced obesity on degradation of miR-17-5p *via* activation of the ER-stress mediators including the inositol requiring enzyme 1α (IRE1α). The authors also demonstrate that inhibiting ER-stress can restore miR-17-5p and TXNIP levels and hence inflammation back to comparable levels seen in normal controls.

Innovations and breakthroughs

The results of their study delineate the contribution of Müller cells, main glia in the retina in palmitate-mediated retinal inflammation. They identify ER-stress as new therapeutic target that is involved in obesity-induced inflammation in pre-diabetic retinopathy.

Applications

Their results suggest that inhibitors of ER-stress reversed the increase in TXNIP *in vivo* and in Müller cells, the main glia in the retina. The findings of their short-term study support the interventional use of the ER-Stress inhibitor PBA, FDA approved drug with high safety profile. This report should open the door for its future studies in diseases associated with TXNIP-NLRP3 inflammation.

Terminology

MicroRNAs are small non-coding RNAs that contribute to the post-transcriptional regulation of various genes expressions. Inflammasome is a multiprotein oligomer responsible for the induction of inflammatory process. Unfolded protein response (UPR) is unfolded protein response, an adaptive mechanism to resolve and slow down protein processing. ER-stress is when the endoplasmic reticulum capacity to deal with UPR is overwhelmed then stress markers such as ATF6, PERK and IRE1α are expressed.

Peer-review

The paper is interesting.

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ORIGINAL ARTICLE

Case Control Study

Association of *NFKB1* gene polymorphism (rs28362491) with levels of inflammatory biomarkers and susceptibility to diabetic nephropathy in Asian Indians

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Abstract

AIM

To investigate the association of *NFKB1* gene -94 ATTG insertion/deletion (rs28362491) polymorphism with inflammatory markers and risk of diabetic nephropathy in Asian Indians.

METHODS

A total of 300 subjects were recruited (100 each), normoglycemic, (NG); type 2 diabetes mellitus (T2DM) without any complications (DM) and T2DM with diabetic nephropathy [DM-chronic renal disease (CRD)]. Analysis was carried out by polymerase chain reaction-restriction fragment length polymorphism and ELISA. Pearson's correlation, analysis of variance and logistic regression were

used for statistical analysis.

RESULTS

The allelic frequencies of -94 ATTG insertion/deletion were 0.655/0.345 (NG), 0.62/0.38 (DM) and 0.775/0.225 (DM-CRD). The -94 ATTG ins allele was associated with significantly increased levels of urinary monocyte chemoattractant protein-1 (uMCP-1); uMCP-1 (P = 0.026) and plasma tumor necrosis factor-alpha (TNF-α); TNF-α (P = 0.030) and almost doubled the risk of diabetic nephropathy (OR = 1.91, 95%CI: 1.080-3.386, P = 0.025).

CONCLUSION

-94 ATTG ins/ins polymorphism might be associated with increased risk of developing nephropathy in Asian Indian subjects with diabetes mellitus.

Key words: Diabetic nephropathy; Inflammation; *NFKB1* -94 ATTG ins/del polymorphism; Urinary monocyte chemoattractant protein-1; Tumor necrosis factor-alpha

Core tip: Type 2 diabetes mellitus (T2DM) is considered as long standing inflammatory disease. Diabetic nephropathy (DN) is the most common micro-vascular complication of T2DM. Pro-inflammatory cytokines like Monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) plays a crucial role in the pathogenesis of DN. Therefore we investigated -94 ins/del ATTG polymorphism in *NFKB1* gene and its association with the risk of DN in Asian Indians. -94 ins/del ATTG single nucleotide polymorphism was found to increase the urinary MCP-1 and plasma TNF-α levels. Our findings open a new area of research to explore that -94 ins/del ATTG may be considered as genetic markers for early detection of diabetic patients who are at greater risk of development of nephropathy.

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INTRODUCTION

Chronic renal disease (CRD) is an intricate pathological process, often leading to end stage renal disease. The causes of CRD are quite multi-factorial ranging from infections to heredity, but type 2 diabetes mellitus (T2DM) is the major culprit amongst them[1]. In spite of the improvement in our knowledge about the etiopathogenesis of diabetic nephropathy (DN), the intricate mechanisms leading to the development of renal injury from chronic hyperglycemia are not yet fully understood. DN has been considered a micro-vascular complication of hyperglycemia, but various clinical and experimental studies have observed that there is a close link between hyperglycemia, inflammation and oxidative stress (OS)[2]. OS may also be involved in promoting a low grade systemic inflammation in patients with T2DM and vice versa[3]. Nuclear factor-kappa B (NF-κB) activation through hyperglycemia induced OS may lead to increased concentration of inflammatory cytokines[4].

NF-κB was identified as a transcription factor which controls the expression of numerous genes affecting immune response, inflammation, cell-growth control, apoptosis and therefore, is an emerging candidate for studies on the pathogenesis of inflammatory diseases including DN. There are five members of the NF-κB family in mammals: NF-κB1: p105/p50, NF-κB2: p52/p100, RelA: p65, RelB, and c-Rel. The chief form of NF-κB is a hetero-dimer of the p50 and p65/RelA subunits, encoded by the *NFKB1* and *RelA* gene. Normally, inactive NF-κB is found in the cytoplasm bound to IkBs, which are specific inhibitor proteins in cytoplasm. Cell when exposed to a variety of proinflammatory stimuli leads to the quick phosphorylation followed by ubiquitinylation, and finally proteolytic breakdown of I-κB. This causes transfer of NF-κB in nucleus and thus leading to increased transcription of gene[5]. NF-κB transcriptionally regulates many downstream proinflammatory genes, mainly including monocyte chemoattractant protein-1 (*MCP-1*), tumor necrosis factor-alpha (*TNF-α*)[6].

MCP-1 is an important proinflammatory chemokine which affects the recruitment and function of monocyte[7]. MCP-1 is synthesized in response to a various proinflammatory stimuli by kidney cell[8]. A study done by Wada *et al*[9] in 2000 has shown that expression of MCP-1 increases in inflammation induced kidney diseases including DN. Urinary MCP-1 (uMCP-1) is a potential biomarker for renal damage[10]. Hyperglycemia induced secretion of abundant MCP-1 from renal parenchymal cells, attract monocytes into the kidney stimulating myofibroblast-like properties in mesangial cells. Kidney macrophages when exposed to MCP-1 in diabetic milieu promotes activation of macrophage. Thus, leading to release of reactive oxygen species (ROS), various pro-inflammatory cytokines and profibrotic growth factors[11,12]. Thus, resulting in exaggerated inflammation that leads to renal injury through proliferation of myofibroblast, augmented production of extracellular matrix by mesangial cells and fibroblasts.

TNF-α is a well known proinflammatory cytokine associated with systemic inflammation[13,14]. It is produced predominantly by macrophages and monocytes[13,14]. TNF-α acts *via* NF-κB signaling and mediates the transcription of various cytokines performing roles in cell survival, proliferation, inflammatory responses, cell adhesion and inflammation[15]. A study has shown that

there is upregulation of TNF-α expression in glomeruli of diabetic rats[16]. TNF-α is well acknowledged to cause damage to renal cells by enhancing renal hypertrophy, hemodynamic imbalance, albumin permeability[17]. The harmful effects of these responses lead to the development of renal disease in patients with T2DM, hence resulting in the progression of renal failure.

In addition to poor glycemic control, OS and inflammation; genetic factors seem to be main determinants of DN in terms of both occurrence and severity[18]; however the genetic mechanism causing DN is still unexplored. In our knowledge, there is no study available regarding the polymorphisms of *NFKB1* and their correlation with levels of uMCP-1 and plasma TNF-α. We have reported[19] increased uMCP-1, plasma TNF-α levels in subjects with DN when compared to subjects with T2DM without nephropathy and observed a positive correlation between uMCP-1 and plasma TNF-α[20]. We have also highlighted that DN is associated with *TNFA* gene single nucleotide polymorphism (SNP)[20]. In recent times, a new functional *NFKB1* promoter SNP consisting of a insertion/deletion (-94ins/del ATTG) (rs28362491) has been identified which can elicit a regulatory effect on the *NFKB1* gene[21]. Since above mentioned polymorphism has been associated with various inflammatory diseases, autoimmune diseases and cancers[22], therefore, it is worthwhile to further investigate the association of -94 ins/del ATTG *NFKB1* gene SNP with levels of uMCP-1, plasma TNF-α and nephropathy risk in subjects with T2DM.

MATERIALS AND METHODS

Study design

The present study comprises of total 300 subjects visiting Nephrology Outpatient Clinic and Medicine OPD at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Subjects were divided into three groups of 100 each namely; Group 1: Normoglycemic (NG), Group 2: Subjects with T2DM for ⩾ 10 years without nephropathy (DM), Group 3: Subjects with T2DM for ⩾ 5 years with nephropathy (DM-CRD). T2DM was diagnosed according to revised ADA criteria[23]. Detailed clinical history and physical examination were recorded. Blood pressure (BP) of subjects was estimated using sphygmomanometer in the sitting position after a resting period of 10 min. The estimated glomerular filtration rate (eGFR) was measured by Modification of Diet in Renal Disease Abbreviated Equation (MDRD)[24].

The presence of micro-albuminuria in T2DM subjects was detected by Urine Test 11 MAU dipstick (Piramal Diagnostic, sensitivity: 10-15 mg/dL), and all participants having proteinuria and micro-albuminuria were clubbed in Group 3. All participants with nephropathy were in pre-dialysis stage. Normoglycemic (Group 1) subjects were recruited from employees of UCMS and GTB Hospital with the following criteria: (1) they did not have of diabetes mellitus (fasting plasma glucose < 100 mg% or postprandial glucose < 140 mg% or HbA1c < 5.7%) according to ADA criteria; (2) there was no presence of diabetes in their first or second degree relatives; and (3) they had normal BP, with systolic and diastolic BP not > 120 mmHg and 80 mmHg[25].

To circumvent any possible confounding factors, patients having renal disorders (hypertensive nephropathy, chronic glomerular nephritis, chronic interstitial disease, ischemic nephropathy, obstructive nephropathy), acute and chronic infections, congestive heart failure, malignancy and liver disorder were not included into the study. All subjects in Group 3 had retinopathy; but participants with macro-vascular complications like coronary artery disease and stroke were not included into the study. Patients taking renin-angiotension aldosterone system inhibitors, aspirin and vitamin D analogues were advised to discontinue these drugs for a period of a week before inclusion in the study since they have been found to influence the synthesis of uMCP-1 and TNF-α. However, patients were prescribed beta-blockers to control BP in that duration of one week. The Institutional Ethics Committee for Human Research approved the protocol of this study (approval number-UCMS/IEC-HR/2010/10). Prior to the inclusion into the present study, informed written consent was taken from all participants.

Biochemical parameters

Under aseptic conditions fasting venous blood samples were withdrawn and collected into EDTA and fluoride vials. For glycosylated hemoglobin (HbA1c) 200 μL whole blood was preserved at 4 ℃-8 ℃ and processed within one week of collection. Blood samples collected in EDTA vial was subjected to centrifugation at 3000 rpm for 10 min in order to separate the plasma. Early morning first mid-stream urine sample was collected and stored in aliquots at -20 ℃ for estimation of MCP-1, albumin and creatinine.

Routine investigations such as fasting and post-prandial plasma glucose, urea, creatinine and uric acid were carried out using commercially available kits on autoanalyser (Olympus AU-400). HbA1c was estimated by ion-exchange resin chromatography using commercially available kits (Fortress, United Kingdom). Urinary protein excretion was expressed as albumin to creatinine ratio.

Markers of inflammation

uMCP-1 (Weldon, California; sensitivity less than 7.8 pg/mL) and plasma TNF-α (Diaclone, France; sensitivity less than 8 pg/mL) were estimated by commercially available ELISA kit.

DNA extraction and polymorphism genotyping

Cellular DNA of every individual was extracted from 200 μL EDTA-anticoagulated peripheral blood sample by means of DNA isolation kit (Zymo research, United States). The polymerase chain reaction was carried out in Thermocycler (Eppendorf Mastercycler Gradient-5331). In brief, 0.1 μg of DNA was amplified in a reaction mixture

Table 1 The baseline demographic and biochemical parameters in various study groups

Variables	NG (n = 100)	DM (n = 100)	DM-CRD (n = 100)
Age (yr)	46.0 ± 4.0	56.40 ± 3.5	55.7 ± 4.2
Sex ratio (male/female)	52/48	54/46	52/48
Duration of DM (yr)	-	12.7 ± 1.5	8.1 ± 2.3[d]
BMI (kg/m^2)	20.1 ± 1.7	21.1 ± 2.1	21.6 ± 3.4
SBP (mmHg)	118.1 ± 0.5	138.0 ± 2.1[b]	137.7 ± 2.8[b]
DBP (mmHg)	75.2 ± 1.0	81.6 ± 1.9[b]	82.8 ± 0.0[b]
Fasting glucose (mg/dL)	82.4 ± 3.1	153.5 ± 3.5[b]	184.3 ± 9.2[b,d]
Postprandial glucose (mg/dL)	118.2 ± 2.4	201.7 ± 10.1[b]	261.1 ± 12.2[b,d]
HbA1c (%)	5.11 ± 0.46	7.10 ± 0.25[b]	9.16 ± 0.16[b,d]
Urea (mg/dL)	31.5 ± 5.5	30.7 ± 5.8	93.2 ± 4.8[b,d]
Creatinine (mg/dL)	0.83 ± 0.23	0.90 ± 0.20	3.7 ± 1.5[b,d]
Uric acid (mg/dL)	4.2 ± 0.8	4.9 ± 0.6	9.1 ± 0.8[b,d]
eGFR (mL/min per 1.73 m^2)	99.1 ± 0.7	96.4 ± 0.6	51.2 ± 0.9[d]
Urinary albumin/creatinine	-	-	0.42 ± 0.35

[b]Significantly different from Normoglycemic at P < 0.001; [d]Significantly different from diabetic patients without nephropathy at P < 0.001. Data are expressed as mean + SD. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy; BMI: Body mass index; SBP and DBP: Systolic and diastolic blood pressure; eGFR: Estimated glomerular filtration rate.

of 20 μL containing 0.5 μmol/L each of the following primer pairs (Forward 5'-TGGGCACAAGTCGTTTATGA-3' and Reverse 5'CTGGAGCCGGTAGGGAAG-3'). The reaction mixture also contained 0.5 mmol/L (dNTP mix), 2 μL (10 × PCR buffer) and 2.0 units Taq DNA polymerase, 2 mmol/L $MgCl_2$. The PCR protocol consist an initial temperature of 94 ℃ (5 min) followed by 35 cycles of amplification (30 s at 94 ℃, 45 s at 59 ℃, and extension for 1 min at 72 ℃). Final extension step was carried out for 2-min at 72 ℃[22].

For the study of the -94 insertion/deletion ATTG SNP in *NFKB1*, PCR product (281/285 bp) was subjected to fast digestion with restriction enzyme *PfIMI*. PCR products was treated with enzyme *PfIMI* in at 37 ℃ for 1 h and inactivated at 65 ℃ for 20 min. The insertion allele (ins) was cut down into two fragments of 45 bp and 240 bp by *PfIMI* restriction enzyme. But, there was no cleavage at the deletion allele (del) that has only one ATTG at its promoter[22]. The bands of digested products were visualized in 2% agarose gel electrophoresis stained with ethidium bromide.

Statistical analysis

Demographic profiles and routine investigation was compared by χ^2 and Student's t test and one-way ANOVA was used. To associate all the study groups with genotype two-way ANOVA followed by *post-hoc* Tukey's test was used. For association of genotypes with uMCP-1 and plasma TNF-α levels, analysis of variance was used. Logistic regressions was used to evaluate the risk of development of DN at the single SNP level. Power of sample size keeping 5% significance level and 80% power was calculated by genetic power calculator. A P value < 0.05 was considered statistically significant (two-tailed). All statistical tests were performed using SPSS version 20.

Table 2 The genotype and allele frequencies of *NFKB1* gene for -94 insertion/deletion ATTG polymorphism in different study groups

	NG (n = 100) n (%)	DM (n = 100) n (%)	DM-CRD (n = 100) n (%)
ins/ins	41 (41)	38 (38)	61 (61)
ins/del	49 (49)	48 (48)	33 (33)
del/del	10 (10)	14 (14)	06[b] (06)
ins allele	131 (65.5)	124 (62)	155 (77.5)
del allele	69 (34.5)	76 (38)	45 (22.5)

[b]Significantly different from diabetic patients without nephropathy at P < 0.001. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy.

RESULTS

Characteristics of the study population

Biochemical and demographic parameters of the various study groups are shown in Table 1. There was no difference in sex distribution and BMI within all the three study groups. The subjects of Group 2 (DM) and Group 3 (DM-CRD) were older than Group 1 (NG) subjects; however the period of diabetes was more in Group 2 (DM) than Group 3 (DM-CRD) which was as per our selection criteria. Incidence of hypertension was significantly higher in Group 2 (DM) and Group 3 (DM-CRD) participants as suggested by raised SBP and DBP (P < 0.001) when compared to NG. Poor glucose control was observed in DM-CRD as compared to DM as suggested by significantly higher (P < 0.001) fasting, postprandial plasma glucose and HbA1c. Renal function tests suggested that blood urea, plasma creatinine, and uric acid were significantly higher (P < 0.001) and eGFR was decreased (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM).

Distribution of ins/del in study population

The allele frequencies and genotype of the *NFKB1* gene for -94 insertion/deletion ATTG SNP in various study groups are shown in Table 2. The distribution percentage of ins/ins, ins/del, del/del genotypes in Group 1 (NG), Group 2 (DM) and Group 3 (DM-CRD) (expressed in percentage) were 41%, 49% and 10%; 38%, 48% and 14%; and 61%, 33% and 6% respectively. The frequency of del/del genotype was significantly lower (P < 0.001) in Group 3 (DM-CRD) as compared to Group 2 (DM). However, allele frequencies of -94 insertion/deletion ATTG were 65.5%/34.5% in Group 1 (NG), 62%/38% in Group 2 (DM) and 77.5%/22.5% in Group 3 (DM-CRD).

Relationship between the -94 ins/del AGGT SNP with inflammatory markers and disease risk

Correlation of -94 ins/del AGGT SNP with levels of

Table 3 Interaction analysis of -94 ins/del ATTG polymorphism with inflammatory markers

Inflammatory marker	Groups	NG (*n* = 100)	DM (*n* = 100)	DM-CRD (*n* = 100)	*P* value
uMCP-1 (pg/mg creatinine)	Total	130.00 ± 42.22	271.00 ± 120.01	5632.70 ± 1007.20[a,b]	
	del/del	85.1 ± 9.2	200.6 ± 66.5	4609.9 ± 900.6	*P* =
	ins/del	110.9 ± 15.6	278.9 ± 105.9	5879.9 ± 1016.3	0.026
	ins/ins	166.8 ± 26.8	302.2 ± 100.1	6405.1 ± 1550.6	
Plasma TNF-α (pg/mL)	Total	15.55 ± 2.22	16.51 ± 3.75	21.38 ± 3.67[a,b]	
	del/del	8.27 ± 1.06	10.21 ± 1.32	17.31 ± 1.17	*P* =
	ins/del	11.55 ± 0.05	14.05 ± 0.18	19.31 ± 0.44	0.030
	ins/ins	15.08 ± 1.15	16.36 ± 1.20	23.12 ± 0.70	

[a]Significantly different from Normoglycemic at $P < 0.001$; [b]Significantly different from diabetic patients without nephropathy at $P < 0.001$. uMCP-1 levels, plasma TNF-α levels are expressed as mean + SD. NG: Normoglycemic; DM: Diabetes mellitus without nephropathy; DM-CRD: Diabetic nephropathy.

uMCP-1 and plasma TNF-α have been studied and the results are shown in Table 3. The -94 ins allele were associated with increased levels of uMCP-1 ($P = 0.026$) and plasma TNF-α ($P = 0.030$) in the disease study groups, *i.e.*, Group 2 (DM), Group 3 (DM-CRD).

The associations at the level of genotype is shown in Table 4. Highly significant association was observed for -94 ins/del AGGT polymorphism in subjects with Group 3 (DM-CRD) in comparison to Group 1 (NG); $P = 0.022$. In our present study, -94 ins SNP was found to increase risk for the development of DN by 1.91-fold in subjects with diabetes (OR = 1.91, 95%CI: 1.080-3.386, $P = 0.025$).

Table 4 Association between -94 ins/del ATTG polymorphism in the *NFKB1* gene and diabetic nephropathy at the genotype level

Genotype	OR	95%CI	*P* value
DM *vs* NG ref	1.04	0.607-4.987	0.887
DM-CRD *vs* NG ref	1.95	1.101-3.467	0.022
DM-CRD *vs* DM ref	1.91	1.080-3.386	0.025

Ref: Referencegroup.

DISCUSSION

Polymorphism in the NFKB1 promoter region at position -94 ins/del AGGT has been correlated with many long standing inflammatory diseases like autoimmune diseases such as rheumatoid arthritis, asthma, AIDS, cancers and various diabetic complications[26,27]. Our study is the first to report the association of above mentioned polymorphism with DN in North Indian population. In the current study, we observed that the frequency distribution of ins/del is maximum in NG and DM subjects followed by ins/ins, with least distribution of del/del in the same. However the trend was different in DM-CRD subjects with respect to ins/del genotype which was less as compared to ins/ins this group. The frequency of different genotypes observed in the present study were in accordance with studies on NFKB1 polymorphism in healthy volunteer in different ethnic population like Turkish[22], Caucasians[28], English[29], Polish[30]. But our results were not in agreement with healthy Chinese population[28]. When our findings were compared with studies on inflammatory diseases like cancer, they are in accordance with a studies conducted in Asian by Huo *et al*[31] and Zhou *et al*[32]. However our results were in contrast with a genomic study on cancer conducted by Yang *et al*[28] in 2014. The dissimilarity of results could be due to diverse geographical distribution and ethnicity between our study and theirs was different, which could result in diverse genetic background.

Latest evidence has shown that the production of MCP-1 by kidney affected by diabetes along with TNF-α is a major cause of inflammation, renal injury and fibrosis in DN[10,17]. The present study is the foremost one to document the correlation of -94 ins/del AGGT SNP with levels of inflammatory markers namely uMCP-1 and plasma TNF-α in DN from North Indian patients. In our previous study, we have observed that plasma TNF-α and uMCP-1levels were significantly raised in patients with T2DM and so more in patients with DN[19]. To explicate the role of NFKB1 gene SNP in the development of DN, -94 ins/del AGGT SNP were analyzed in various study, *i.e.*, Group 2 (DM) and Group 3 (DM-CRD) and further correlated with measured inflammatory markers like uMCP-1 and plasma TNF-α levels. Interestingly, this study has also shown that ins allele was significantly associated with increased urinary MCP-1 and plasma TNF-α levels in NG as well as patient groups. However, there is no report in literature to compare our results.

A recent study has shown that TNF-α stimulates the MCP-1 production *via* NF-κB signalling pathway in rat astrocyte cultures[33]. TNF-α was found to increase p65 and phosphorylated p65 levels in nuclear extracts of rat astrocytes, hence augmenting MCP-1 levels[33]. This supports our finding that increased levels of TNF-α are associated with increased levels of uMCP-1.

Genetic variations are known to play a vital role in determining risk of DN. A number of studies have investigated the relationship of ins allele of -94 ins/del AGGT polymorphism with various inflammatory diseases. Till date not a single study has tried to evaluate the association between this polymorphism and DN risk. Our study is first to document that patients with T2DM having ins/ins genotype were found to have increased risk of developing nephropathy. Latest studies have reported that p50 null mice have a significantly reduced inflammatory response in various models of inflammation such as asthma[34], arthritis[35], and autoimmune encephalomyelitis[36]. A similar study conducted in sporadic colorectal cancer (CRC)[37] and epithelial ovarian cancer (EOC)[31] has supported

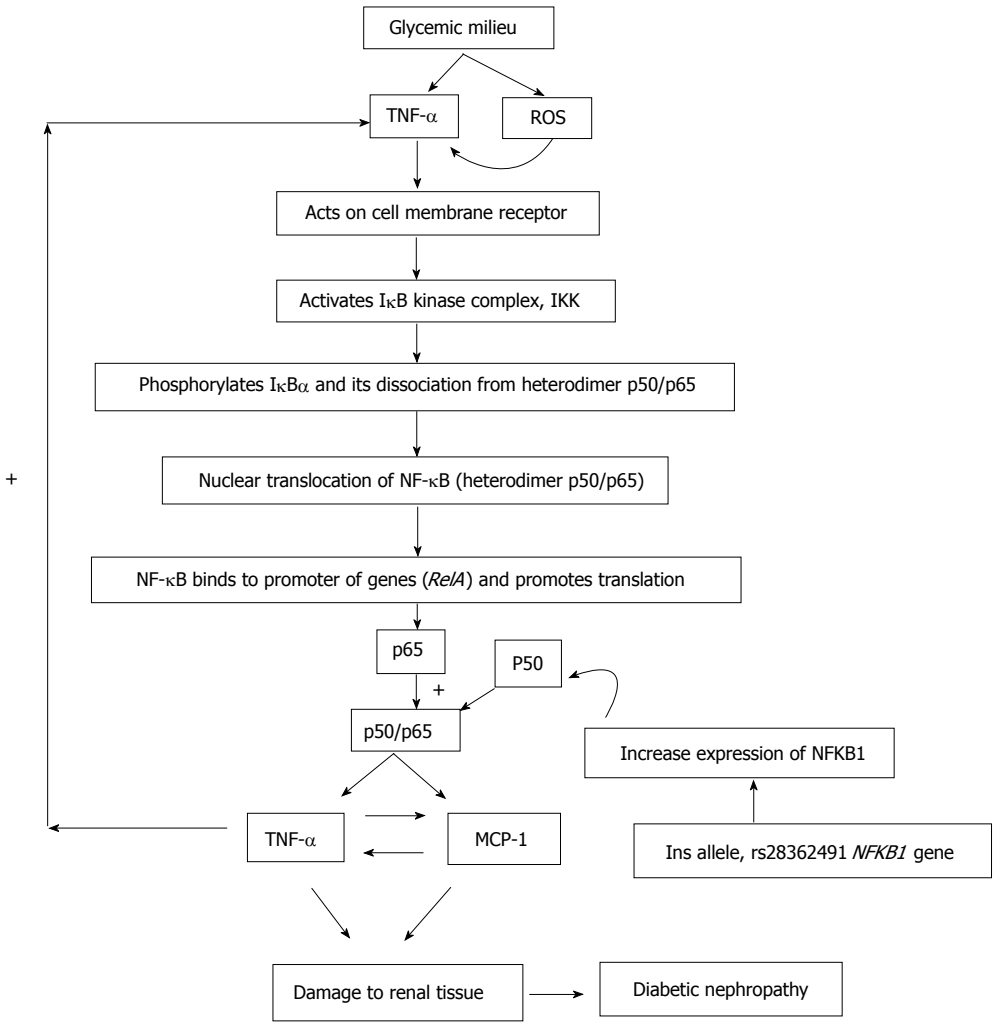

Figure 1 NFKB1 gene and inflammatory markers: Probable mechanisms in the pathogenesis of diabetic nephropathy. Hypoglycemia induced ROS and TNF-α leads to activation of IKK. IKK causes phosphorylation of IκBα bound to p50/p65. Phosphorylated IκBα dissociate from p50/p65 leading to nuclear translocation of unbound heterodimer p50/p65 (NF-κB). Binding of NF-κB to promoter gene causes translation of p65. Ins allele, rs28362491 *NFKB1* gene, if present, causes increase expression of p50. Hence there is increased production of p50/p65 heterodimer complex. This heterodimer acts on its downstream proinflammatory targets viz: MCP-1 and TNF-α leading to its synthesis. MCP-1 is a positive regulator of TNF-α and vice versa. Both MCP-1 and TNF-α causes renal damage leading to development of Diabetic nephropathy. ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-alpha; IKK: IκB kinase complex; MCP-1: Monocyte chemoattractant protein-1.

our findings which suggested that ins/ins genotype contribute to significantly increased risk of CRC and EOC. The probable mechanism of -94 ins/del AGGT polymorphism leading to increased risk of developing DN is explained in Figure 1. In almost all cell types, NF-κB complexes are typically localized in the cytoplasm where they bind to IKB inhibitory proteins. However, stimulation with hyperglycemia induced ROS and TNF-α leads to rapid phosphorylation of IKB *via* I-κB kinases complex which is then degraded by ubiquitin-proteosome pathway. On the other hand, simultaneously -94 ins/del AGGT polymorphism might lead to increased synthesis of p50 mRNA. Hence there will be increased production of p50/p65 hetrodimer complex which is a well known proinflammatory molecule, since p50/p65 hetrodimer acts on its downstream proinflammatory targets *viz*: MCP-1 and TNF-α, leading to over production of MCP-1 and TNF-α. Thus, there occurs a viscous cycle, *i.e*., MCP-1 is a positive regulator of TNF-α and vice versa. The above mentioned probable hypothesis might lead to increased risk of developing renal damage in T2DM. However results of a recent study from China[38] in bladder cancer is in contradiction to our findings which could be due to ethnic and geographical differences. Furthermore, the sample size of our study was fairly small than aforementioned bladder cancer study.

The results of the current study suggest that the NFKB1 promoter -94 ins/del AGGT SNP is associated with increased possibility of developing nephropathy in patients with diabetes. This SNP may be considered as genetic markers for susceptibility to develop nephropathy in patients with T2DM. The limitation of the study is the small sample size. Therefore, further evaluation is necessary in big sample size to look for the possibility of this polymorphisms as potential genetic markers in the near future. This would help to identify patients with type 2 diabetics who may be at higher risk of developing nephropathy.

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COMMENTS

Background

Type 2 diabetes mellitus (T2DM) is considered as a long standing inflammatory disease. Nuclear factor-kappa B (NF-κB) controls the expression of numerous genes affecting inflammation, immune response. Immunogenic and inflammatory cytokines like monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) plays a crucial role in the pathogenesis of micro-vascular complication of T2DM, *i.e.*, diabetic nephropathy (DN) and clinical outcome.

Research frontiers

In spite of the present advances in our knowledge about the etiopathogenesis of DN, the intricate mechanisms leading to the development of renal injury from chronic hyperglycemia are not yet fully understood. *NFKB1* promoter polymorphism -94 ins/del ATTG has been associated with inflammatory diseases, autoimmune diseases and cancers. However, its role in the development of T2DM and DN has not been explored till date. The authors hypothesized that the -94 ins/del ATTG polymorphism would affect the levels of urinary MCP-1 and plasma TNF-α and therefore might be culprit in developing DN.

Innovations and breakthroughs

The authors have recently reported that -94 ATTG ins allele was associated with significantly increased levels of urinary MCP-1, plasma TNF-α and was found to increase risk for the development of DN by 1.91-fold in subjects with diabetes.

Applications

-94 ins/del AGGT polymorphisms can be considered as genetic marker for identifying those more susceptible and provide suitable interventions to delay the progression of DN. This study provides a ground for the development of newer anti-inflammatory therapeutic agents that may have potential to affect primary mechanisms contributing to the pathogenesis of DN.

Terminology

DN: Diabetic nephropathy; NF-κB: Nuclear factor-kappa B; *NFKB1*: Nuclear factor-kappa B1 gene; T2DM: Type 2 diabetes mellitus; TNF-α: Tumor necrosis factor-alpha; uMCP-1: Urinary Monocyte chemoattractant protein-1.

Peer-review

The manuscript is well informative.

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ORIGINAL ARTICLE

Case Control Study

Exercise-induced albuminuria *vs* circadian variations in blood pressure in type 1 diabetes

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Informed consent statement: All participants and their parents or guardians (since many were adolescents) provided informed written concern prior to study enrollment.

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Data sharing statement: Data are available from the corresponding author upon request at sobngwieugene@yahoo.fr.

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Abstract

AIM

To investigated the relationship between exercise-induced ambulatory blood pressure measurement (ABPM) abnormalities in type 1 diabetes mellitus (T1DM) adolescents.

METHODS

We conducted a case-control at the National Obesity Center of the Yaoundé Central Hospital, Cameroon. We compared 24 h ABPM and urinary albumin-to-creatinine ratio (ACR) at rest and after a standardized treadmill exercise between 20 Cameroonian T1DM patients and 20 matched controls. T1DM adolescents were aged 12-18 years, with diabetes for at least one year, without proteinuria, with normal office blood pressure (BP) and renal function according to the general reference

population. Non-diabetic controls were adolescents of general population matched for sex, age and BMI.

RESULTS

Mean duration of diabetes was 4.2 ± 2.8 years. The mean 24 h systolic blood pressure (SBP) and diastolic blood pressure (DBP) were respectively 116 ± 9 mmHg in the diabetic group *vs* 111 ± 8 mmHg in the non-diabetic (P = 0.06), and 69 ± 7 mm Hg *vs* 66 ± 5 mm Hg (P = 0.19). There was no difference in the diurnal pattern of BP in diabetes patients and non-diabetic controls (SBP: 118 ± 10 mmHg *vs* 114 ± 10 mmHg, P = 0.11; DBP: 71 ± 7 mmHg *vs* 68 ± 6 mmHg, P = 0.22). Nighttime BP was higher in the diabetic group with respect to SBP (112 ± 11 mmHg *vs* 106 ± 7 mmHg, P = 0.06) and to the mean arterial pressure (MAP) (89 ± 9 mmHg *vs* 81 ± 6 mmHg, P = 0.06). ACR at rest was similar in both groups (5.5 mg/g *vs* 5.5 mg/g, P = 0.74), but significantly higher in diabetes patients after exercise (10.5 mg/g *vs* 5.5 mg/g, P = 0.03). SBP was higher in patients having exercise-induced albuminuria (116 ± 10 mmHg *vs* 108 ± 10 mmHg, P = 0.09).

CONCLUSION

Exercise-induced albuminuria could be useful for early diagnosis of kidney damage in adolescents with T1DM.

Key words: Albuminuria; Blood pressure; Ambulatory blood pressure measurement; Exercise; Type 1 diabetes

Core tip: Diabetic nephropathy (DN) is a major complication of type 1 diabetes mellitus (T1DM). Therefore, strategies for early detection are of critical importance. Ambulatory blood pressure measurement is useful for detection of precocious abnormalities in the occurrence of DN and exercise-induced albuminuria has been proposed as a potential predictor of DN. Our study therefore aimed to investigate the relationship between exercise-induced albuminuria and ambulatory blood pressure measurement abnormalities in T1DM Cameroonian adolescents. We found that T1DM patients had higher nocturnal and 24 h blood pressure figures than non-diabetics suggesting that exercise-induced albuminuria could be useful early detection of diabetes kidney injuries in T1DM.

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INTRODUCTION

Diabetes nephropathy is the major life-threatening complication of type 1 diabetes mellitus (T1DM)[1,2]. Abnormal albumin excretion has been shown to predict the development of clinically significant nephropathy in T1DM. Indeed, persistent minimal elevation of albuminuria at rest predicts the development of more severe proteinuria and clinical diabetic nephropathy, which frequently progresses to renal failure[3]. In T1DM, nephropathy develops in 30% to 40% of cases and impaired renal function or end-stage kidney disease affect up to a third of patients[4]. Thus, strategies for early detection and for preventative interventions are of critical importance since interventions at these late stages of disease may only slow but not completely arrest the inexorable progression towards renal failure[5,6]. In this direction, it has been shown that physical exercise can stimulate albuminuria in diabetes patients and can be a useful provocative test to detect early renal abnormalities[7]. However, there is still limited evidence on its value for early detection of renal disease in T1DM.

Previous studies has proven that during exercise, urinary albumin excretion rate is more increased in long term T1DM patients thus, at risk of developing diabetes nephropathy than in general population[8]. In the contrary, some evidence suggest that the level of albumin excretion during exercise is related to the quality of metabolic control; for example, exercise-induced microalbuminuria is more pronounced in newly diagnosed patients, and this abnormality is reversed by insulin treatment. Exercise-induced microalbuminuria generally is not well correlated with the duration of disease and does not predict clinical nephropathy[9]. On the other hand, the contribution of night-time blood pressure (BP) on the onset of nephropathy in diabetic patients is now established[10]. Therefore ambulatory blood pressure monitoring (ABPM) could be proposed as an useful tool for early detection of diabetic nephropathy[11,12]. This study aimed to investigate the relationship between exercise-induced albuminuria and ABPM abnormalities in early detection of diabetic nephropathy in adolescents with T1DM from Cameroon.

MATERIALS AND METHODS

Study subjects

This case-control study was carried out at the National Obesity Center of the Yaoundé Central Hospital, the reference diabetes center in the town. Our population was made of two groups, T1DM adolescents and non-diabetic controls. T1DM patients were aged 12-18 years, with diabetes for at least one year; without proteinuria, with normal office BP and renal function according to the general reference population. Non-diabetic controls were adolescents of general population matched for sex, age and BMI. We excluded patients and controls with an important night activity, those receiving drugs for hypertension or any other drugs able to modify albuminuria, those with contra-indication to exercise or presenting signs of urinary tract infection as well as those having fever and pregnant women.

Procedure and investigations

The procedure was made of an inclusion visit and two exploration visits. Within 2 wk following an information visit, for all eligible participants, we performed a careful clinical exam including BP measurement and a urinary dipstick. We enrolled 40 participants, 20 in each group.

All exploration visits were conducted in the morning between 8:00 and 10:00. After arrival, participants were invited to stay in sitting position for at least five minutes. Then, clinical measurement of BP was done three time using an automated sphygmomanometer Omron HEM-705 CP (Omron Corporation, Tokyo, Japan) placed on the left arm raised itself at the heart level. The average of three measures was considered for analysis. Weight and height were respectively valued to the nearest 0.5 unit using a mechanical scale and a measuring rod and body mass index (BMI in kg/m²) calculated as *weight* (cm)/[*height* (m) × *height* (m)]. A dipstick was done to assess proteinuria and considered positive for at least 1+.

ABPM was carried out on twenty four hours using an automatic portable, light weight monitor device the i-MAPA® CE 004 1.1 TM (High-tech Medical St Louis, Paris) which performs measurements every 15 min during daytime (07:00 to 22:00) and twice an hour during night time defined from 22:00 to 07:00. Device was activated and the two first measures performed in the laboratory to ensure functionality. Detailed information on the operation and use of the device were then given to the participant who then returned to his daily activities. At least 70% of valid measurements were considered for interpretation.

The exercise protocol was developed according to the Hierarchy of individual calibration levels for heart rate and accelerometry to measure physical activity[13]. It was made of 1 km race on a treadmill at 5.8 km/h and was divided in two phases. The first phase was made of a 3 min gathering speed up to 3.2 km/h, followed by an acceleration of 0.33 km/h every 6 min. The second phase was a walking step between 5.2-5.8 km/h on the treadmill.

Albuminuria was calculated using albumin-to-creatinine ratio in order to avoid effect of exercise on urinary concentration and expressed in mg/g. First void urine collection was used for rest albuminuria and a random sample urine was collected within the 20 min following physical exercise to measure exercise-induced albuminuria. Albuminuria or exercise-induced albuminuria was diagnosed on the basis of a urinary albumin excretion rate greater than 20 but less than 200 mg/g[14]. Adverse events such as hypoglycemia during physical exercise or exercise intolerance, were closely monitored.

Statistical analysis

Data acquisition was done by Epi-data 3.1 software and statistical analysis was performed using Stata 12.0 software. Continuous variables are expressed as means with standard deviation (SD) where appropriate, and categorical variables as count (percentage). The Spearman rank coefficient was used to test correlations. The χ^2 test and Mann-Whitney rank sum test were used to test associations between qualitative variables and difference between two respectively. A P value $\leqslant 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Mr. Sontsa.

Table 1 Ambulatory blood pressure measurement of the diabetes and non-diabetes patients

Variables	Type 1 diabetic patients (n = 20)	Non-diabetic patients (n = 20)	P value
24 h BP			
SBP	116 ± 9	111 ± 8	0.06
DBP	69 ± 7	66 ± 5	0.19
PP	48 ± 8	45 ± 5	0.11
Diurnal BP			
SBP	118 ± 10	114 ± 10	0.11
MAP	92 ± 7	89 ± 7	0.15
DBP	71 ± 7	68 ± 6	0.22
Nocturnal BP			
SBP	112 ± 11	106 ± 7	0.06
MAP	85 ± 9	81 ± 6	0.06
DBP	64 ± 9	60 ± 6	0.11

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial blood pressure; PP: Pulse pressure.

RESULTS

General characteristics

We enrolled 40 participants, 24 males, average age of 16 ± 2 years. The mean BMI of diabetes patients was 22.6 ± 2.9 kg/m² *vs* 22.7 ± 3.3 kg/m² for non-diabetic. Average duration of diabetes was 4.2 ± 2.8 years with mean glycated hemoglobin of 9.9 ± 2.8. Nine diabetes patients had a family history of hypertension *vs* six in the non-diabetic group.

ABPM measurement of study population

Diabetes participants had lightly higher BP values compared to non-diabetic on every component (Table 1). Thus, 24 h SBP measurement in the diabetic group was 116 ± 9 mmHg *vs* 111 ± 8 mmHg for non-diabetics at borderline of significance (P = 0.06) while difference in DBP of two groups was non-significant (69 ± 7 mmHg *vs* 66 ± 5 mmHg; P = 0.19). In keeping with that, diurnal BP figures were slightly higher in the diabetic group but with a non-significant difference (SBP: 118 ± 10 mmHg *vs* 114 ± 10 mmHg, P = 0.11; DBP: 71 ± 7 mmHg *vs* 68 ± 6 mmHg; P = 0.22). One important finding was the elevated night time BP in diabetes adolescents with a borderline significance for SBP (112 ± 11 mmHg *vs* 106 ± 7 mmHg, P = 0.06) and MAP (85 ± 9 mmHg *vs* 81 ± 6 mmHg, P = 0.06).

Urinary albumin excretion of study population

In adolescents with diabetes, 06/20 (30%) developed abnormal exercise-induced albuminuria but none in the group of adolescents without diabetes. Urinary albumin

Table 2 Comparison of blood pressure values for albuminurics and non albuminurics patients

	UAE < 20 mg/g	UAE > 20 mg/g	*P* value
24 h BP			
SBP	113 ± 9	119 ± 10	0.14
DBP	67 ± 6	70 ± 8	0.51
Diurnal BP			
SBP	116 ± 10	120 ± 10	0.32
DBP	70 ± 5	72 ± 8	0.51
Nocturnal BP			
SBP	108 ± 10	116 ± 10	0.09
DBP	61 ± 8	66 ± 9	0.17

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; UAE: Urinary albumin excretion.

excretion at rest was similar in both groups (5.5 mg/g *vs* 5.5 mg/g, $P = 0.74$). After exercise, we found a significant increase in urinary albumin excretion in diabetes patients as compared to non-diabetics (10.5 mg/g *vs* 5.5 mg/g, $P = 0.03$).

Relation between BP profile and albuminuria at rest and after exercise

We compared diabetes adolescents presenting exercise-induced albuminuria after exercise to those without albuminuria (Table 2). We found that diabetes patients with exercise-induced albuminuria had higher but non-significant nighttime SBP figures than those exercise-induced albuminuria (116 mmHg *vs* 108 mmHg, $P = 0.09$) while DBP were similar. In contrast, 24 h SBP and DBP were similar in both as well as diurnal SBP and DBP.

DISCUSSION

This study aimed to investigate the relationship between exercise-induced albuminuria and circadian BP abnormalities revealed by ABPM in non proteinuric T1DM adolescents. In order to achieve this objective, we compared young T1DM patients to non-diabetic matched controls. We found that nocturnal SBP of diabetic patients was slightly higher than that of non-diabetics as well as 24 h SBP with borderline significance. Most T1DM studies on albuminuria disease have been done in Caucasians[14-17]. This study confirms these findings in Africans. This increase in nocturnal SBP values and 24 h SBP already found by others studies suggest the existence in this group of probable subclinical kidney injuries. Indeed, it was demonstrated that diabetes patients with kidney injury or subclinical diabetic nephropathy had a tendency to higher BP than the general population[14-18]. Similarly, diabetes patients in our study have a tendency to increased nocturnal BP figures in comparison to non-diabetics leading to a reduction in the difference of day-night BP evaluated by dipping[19,20]. This anomaly is found more frequently in diabetes patients compared than in the general population and is attributed to the presence of kidney damage, still subclinical, but already leading to an increase in renal and cardiovascular risk[18]. Thus, the studies comparing individuals with impaired nocturnal decline in BP and those with normal nocturnal BP have revealed that individuals with insufficient decrease of BP and therefore higher values of BP during the night will present in future monitoring a more rapid degradation of renal function marked by a significant decrease in creatinine clearance[21]. In the same sense, these studies did not find any difference between daytime BP as well as diastolic BP which was also to be the case in our study where daytime BP were similar in both groups of participants[18,20]. However, unlike these studies, we found 24 h BP figures slightly higher in diabetes individuals but still of borderline significance. This could be attributed to the impact of nighttime BP on the 24 h BP and would be a reflection of the nocturnal difference since for similar diurnal BP, if the nocturnal BP is elevated in one group, then it becomes logical that the 24 h BP which is the average daytime and nighttime BP appears to be also more elevated.

Secondly, our study showed that for similar or even identical values of albuminuria at rest, diabetes patients having an increase in nocturnal BP and therefore probable subclinical kidney injuries had a significantly increase in exercise-induced albuminuria in comparison to non-diabetic individuals. This suggests that exercise-induced albuminuria increases with the existence of renal alterations revealed by abnormal nocturnal BP and therefore could be used to detect patients with these abnormalities. This finding support the assumption that exercise-induced albuminuria could serve as a marker of early diabetic renal injuries and allow detection or at least help to suspect the existence of subclinical diabetic nephropathy still undetectable by albuminuria at rest. This had been suggested in 1995 by O'Brien who found during a prospective follow-up on a half-decade that patients having abnormal exercise-induced albuminuria were those who would develop a clinical albuminuria at rest and therefore faster diabetic nephropathy[22-25]. But to the best of our knowledge, nobody has so far studied the relationship between exercise-induced albuminuria and nocturnal abnormalities of BP in type 1 diabetes patients. This first finding then proves very encouraging since it opens the way to new opportunities and show new research fields to explore.

Finally, we compared the diurnal and nocturnal BP values of patients who developed exercise-induced albuminuria to those of other participants without this abnormality. We found that patients with exercise-induced albuminuria had higher non-significant figures of BP during the night than those without this abnormality. These data support the hypothesis emitted above that exercise induced-albuminuria could be used to identify T1DM patients with abnormal nocturnal BP and therefore at risk of developing diabetic nephropathy or already presenting subclinical damage due to diabetic nephropathy. However, these findings casually refer to other studies on the subject with larger population study

and ideally with a prospective follow-up in order to clearly establish the link between exercise-induced albuminuria and renal prognosis and cardiovascular evaluated by circadian BP on ABPM and especially nocturnal BP abnormalities in T1DM[26-28].

In summary, T1DM patients having an increase in nocturnal BP exhibit an increase exercise-induced albuminuria and patients developing abnormal exercise-induced albuminuria have higher figures of nocturnal BP than others. These findings strongly suggest that exercise-induced albuminuria could to be use identify diabetes patients with subclinical renal damage, therefore it would be useful in the early diagnosis of nephropathy in T1DM.

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COMMENTS

Background

Nocturnal abnormalities of blood pressure are correlated with incipient diabetes nephropathy in type 1 diabetes adolescents, but relation with exercised induced-albuminuria has not been investigated yet. Few studies have been conducted on diabetic nephropathy in Africans adolescents.

Research frontiers

Studies on diabetic nephropathy in Africans adolescents are scarce. These data are important to determine the tie between exercise-induced albuminuria and nocturnal blood pressure abnormalities in type 1 diabetes adolescents and the possibility to use it as an earlier marker for diabetes nephropathy.

Innovations and breakthroughs

The authors confirm data of Caucasians studies suggesting that most type 1 diabetes adolescents developed diabetes nephropathy after five years. This study was the first investigating the relationship between exercise-induced albuminuria and ambulatory blood pressure measurement measurements in type 1 diabetes adolescents in the search of early markers of diabetic nephropathy.

Applications

This study shows that there is a relation between exercised-induced albuminuria and nocturnal abnormalities of circadian blood pressure suggesting that exercised-induced albuminuria could be useful as clinical marker for blunted nighttime in type 1 diabetes adolescents.

Peer-review

This is a nice study and well done, the topic is clear and the conclusion is novel.

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EVIDENCE-BASED MEDICINE

Fuzzy expert system for diagnosing diabetic neuropathy

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Abstract

AIM

To design a fuzzy expert system to help detect and diagnose the severity of diabetic neuropathy.

METHODS

The research was completed in 2014 and consisted of two main phases. In the first phase, the diagnostic parameters were determined based on the literature review and by investigating specialists' perspectives (n = 8). In the second phase, 244 medical records related to the patients who were visited in an endocrinology and metabolism research centre during the first six months of 2014 and were primarily diagnosed with diabetic neuropathy, were used to test the sensitivity, specificity, and accuracy of the fuzzy expert system.

RESULTS

The final diagnostic parameters included the duration of diabetes, the score of a symptom examination based on the Michigan questionnaire, the score of a sign examination based on the Michigan questionnaire, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria. The output variable was the severity of diabetic neuropathy which was shown as a number between zero and 10, had been divided into four categories: absence of the disease, (the degree of severity) mild, moderate, and severe. The interface of the system was designed by ASP.Net (Active Server Pages Network Enabled Technology) and the system function was tested in terms of sensitivity (true positive rate) (89%), specificity (true negative rate) (98%), and accuracy (a proportion of true results, both positive and negative) (93%).

CONCLUSION

The system designed in this study can help specialists

and general practitioners to diagnose the disease more quickly to improve the quality of care for patients.

Key words: Expert systems; Fuzzy logic; Artificial intelligence; Diabetes mellitus; Diabetes complications; Diabetic neuropathies

Core tip: In this study, an expert system was designed for diagnosing diabetic neuropathy. This system can help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. Even general practitioners can use this system in remote areas to improve the quality of care for patients with diabetes. With it, patients will no longer need to undertake complex procedures, and the care plan can be applied at the right time.

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INTRODUCTION

One of the biggest challenges currently experienced by healthcare organizations is the increasing burden of chronic diseases posing serious threats to public health in developing countries[1]. Diabetes is one of the world's most common and costly chronic diseases, and the number of patients suffering from diabetes has been showing an increasing trend in many countries[2]. This can be attributed to population growth, aging, urbanization, prevalence of obesity, and a sedentary lifestyle[2,3]. Long-term complications of diabetes develop gradually and might be disabling or life-threatening - for example, vascular and tissue injuries caused by the progression of diabetes can lead to serious complications, such as retinopathy, nephropathy, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, metabolic disease, and diabetic foot ulcer[4,5]. However, the most common complication of diabetes is impairment of the peripheral neural system, which is known as diabetic neuropathy and a major problem with different signs and symptoms. Compared with other diabetes complications, it is one of the first reasons for hospitalizing patients with diabetes[6]. The severity of pain, decreased or lack of sensation, increased risk of foot ulceration, and amputation are the consequences of diabetic neuropathy[7].

Diabetic peripheral neuropathy is usually seen in more than 10% of patients with type Ⅱ diabetes. Early diagnosis and treatment is the first step to reduce the incidence of foot ulcers and amputations[8]. The main cost of this disease is related to organ amputation. The risk of lower extremity amputation in patients is significantly high in case of this disease. Nevertheless, almost 85% of amputations are preventable by early detection of the disease, early intervention, good control of diabetes, and patient education[9]. Moreover, several studies show that neuropathy may negatively affect the quality of life for patients with diabetes[10,11].

Owing to the high prevalence of neuropathy among patients with diabetes, it is necessary to conduct annual screening and further evaluation as well as to devise a plan for managing the disease. However, one of the major problems associated with the diagnosis of diabetic neuropathy is the lack of a reliable clinical scale for grading the severity of the disease[12]. A variety of methods are used to detect peripheral neuropathy. These include the nerve conduction velocity test, the vibration perception threshold, the monofilament test, the clinical neuropathy examination, the Toronto clinical scoring system, and the Michigan neuropathy screening instrument (MNSI)[13]. Other than clinical examination, laboratory tests, such as haemoglobin A1c level, fasting blood sugar, and oral glucose tolerance test, along with risk factors like age, sex, renal disease, and smoking need to be considered[14].

It is notable that the boundary between illness and health is not clear in diabetic neuropathy, and it is difficult to express clinical diagnosis as the lack of or the existence of the disease. Since the disease develops on a continuous basis, two-valued logic cannot be used to express this continuity anymore[6]. Therefore, new methods for diagnosing the disease have been considered[15]. Among these methods, special attention has been paid to the development of information technology applications, decision support systems, and fuzzy expert systems[16,17]. The fuzzy expert system is a new version of expert systems that uses fuzzy logic for data processing. In a fuzzy expert system, the inference is conducted by a set of membership functions and fuzzy rules rather than by the rules of two-valued logic[18]. The Fuzzy expert systems are used to describe uncertain phenomena because real-world phenomena are much more complex than an exact and absolute description[19,20]. The ability to implement human science through specific linguistic concepts and fuzzy rules, non-linearity, adaptability of these systems, and the level of accuracy are the most important features of these systems[21]. Although fuzzy expert systems have been designed for different purposes in the healthcare setting, only a few studies have focused on the use of these systems with regard to the diagnosis of diabetic neuropathy[22].

MATERIALS AND METHODS

Objective

To design a fuzzy expert system to categorize the severity of diabetic neuropathy based on clinical exa-

minations and results of laboratory tests.

Setting, design, and sample size

This study was completed in 2014. The study consisted of two main phases. In the first phase, the parameters required for the diagnosis of diabetic neuropathy were determined on the basis of the literature review[23,24]. These parameters formed a questionnaire to investigate specialists' views about the importance of each of them. In the second phase, the system was tested by using real data. In the first phase, eight endocrinologists participated in the study. Owing to the limited number of specialists, no sampling method was applied in this phase. In the second phase, 244 medical records were identified from a database located in an endocrinology and metabolism research centre. These records were related to those patients who visited the centre during the first six months of 2014 and who were primarily diagnosed with diabetic neuropathy.

Methods for data collection and distribution

The questionnaire was distributed among the specialists by one the researchers (MRK), and their views on the importance of the diagnostic parameters were investigated. In second phase, a form was used to extract the required data from the medical records.

Development of the questionnaire

As noted before, the questionnaire was designed based on the literature review[23,24]. It comprised two parts: The first part included the specialists' demographic information, such as age, gender, and work experience; the second part was designed based on a five-point Likert scale (5 = very important, 4 = important, 3 = relatively important, 2 = less important, 1 = unimportant) and consisted of 15 questions to identify the degree of importance of each diagnostic parameter. The face and content validity of the questionnaire was approved by experts in the field of endocrinology. Its reliability was confirmed by using the test-retest method ($\alpha = 0.9$).

Statistical analysis

A data analysis was performed by using SPSS (version 20.0) software, and parameters with a mean score of less than three were excluded to facilitate the process of writing fuzzy rules. To test the system, the sensitivity, specificity, and accuracy of the fuzzy expert system were measured and compared with the final diagnosis recorded in the database. Cohen's kappa coefficient and the receiver operating characteristic (ROC) curve were used to report data.

Participants and recruitment

Before conducting the research, the approval of an institutional review board was obtained. In the first phase, the target population comprised endocrinologists working in an endocrinology and metabolism research centre. They were contacted by one of the researchers (MRK) and the research facilitator (MM), and were invited to take part in the study. Their participation in the research was completely voluntary. Regarding the medical records, patient identities were excluded and only the required data was extracted so that it can be used in the process of evaluation.

RESULTS

Participants

As noted before, the first part of the questionnaire included the participants' demographic information. According to the results, most of the participants were men (n = 5, 62.5%) aged between 30-50 years. The highest frequency (n = 3, 37.5%) was related to the age group of 46-50 years and the specialists with more than 16 years of work experience.

Diagnostic parameters for diagnosing diabetic neuropathy

The second part of the questionnaire was related to the diagnostic parameters required for diagnosing diabetic neuropathy. This part included the duration of diabetes, the symptom assessment based on MNSI, the sign examination based on MNSI, and the related laboratory tests. Table 1 presents the specialists' views in relation to the importance of the aforementioned diagnostic parameters.

As Table 1 shows, from the specialists' point of view, the most important diagnostic parameters were the duration of diabetes (4.88 ± 0.35), the glycolysis haemoglobin level (4.50 ± 0.75), and the score of the sign examination based on the Michigan questionnaire (4.38 ± 0.51). The lowest degree of importance (2.13 ± 0.83) was related to the amount of phosphorus in blood. After determining the diagnostic parameters of diabetic neuropathy, the semantic network of the expert system was drawn (Figure 1).

Designing a fuzzy expert system

As can be seen in the above figure, the ultimate goal, namely diagnosing diabetic neuropathy, is shown in the centre, and the diagnostic parameters are in the leaf nodes. In order to design the fuzzy expert system, all input variables were fuzzified based on membership functions. The system had seven input variables: The duration of diabetes, the score of the symptom examination based on the Michigan questionnaire, the score of the sign examination based on the Michigan questionnaire, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria. The system also had one output variable, which was the severity of diabetic neuropathy. The rules of the expert system were written based on the semantic network, consulting a specialist, and giving the same weight to all rules. The inference engine of the system was designed by using the Mamdani inference method. Figure 2 provides

Table 1 The degree of importance of the diagnostic parameters for diagnosing diabetic neuropathy from the specialists' perspectives

Degree of importance	Unimportant (1)	Less important (2)	Relatively important (3)	Important (4)	Very important (5)	Mean ± SD
Duration of diabetes	0	0	0	1 (12.5%)	7 (87.5%)	4.88 ± 0.35
Symptom assessment based on MNSI	0	0	1 (12.5%)	5 (62.5%)	2 (25%)	4.13 ± 0.64
Sign examination based on MNSI	0	0	0	5 (62.5%)	3 (37.5%)	4.38 ± 0.51
HbA1c	0	0	1 (12.5%)	2 (25%)	5 (62.5%)	4.50 ± 0.75
CBC	1 (12.5%)	3 (37.5%)	4 (50%)	0	0	2.38 ± 0.74
FBS	0	0	0	6 (75%)	2 (25%)	4.25 ± 046
ESR	1 (12.5%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	0	2.52 ± 092
Oral GTT	1 (12.5%)	4 (50%)	1 (12.5%)	2 (25%)	0	2.50 ± 1.06
Albuminuria	0	1 (12.5%)	1 (12.5%)	4 (50%)	2 (25%)	3.88 ± 0.99
TSH	2 (25%)	1 (12.5%)	3 (37.5%)	2 (25%)	0	2.63 ± 1.18
B12 Vitamin	2 (25%)	1 (12.5%)	1 (12.5%)	4 (50%)	0	2.88 ± 1.35
BUN	1 (12.5%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	0	2.38 ± 0.91
BCr	0	1 (12.5%)	2 (25%)	5 (62.5%)	0	3.50 ± 0.75
Calcium	2 (25%)	1 (12.5%)	4 (50%)	1 (12.5%)	0	2.50 ± 1.06
Phosphorus	2 (25%)	3 (37.5%)	3 (37.5%)	0	0	2.13 ± 0.83

BCr: Blood Creatinine; BUN: Blood urea nitrogen; TSH: Thyroid-stimulating hormone; GTT: Glucose tolerance test; ESR: Erythrocyte sedimentation rate; MNSI: Michigan Neuropathy Screening Instrument; HbA1c: Hemoglobin A1c; CBC: Complete blood count; FBS: Fasting blood sugar.

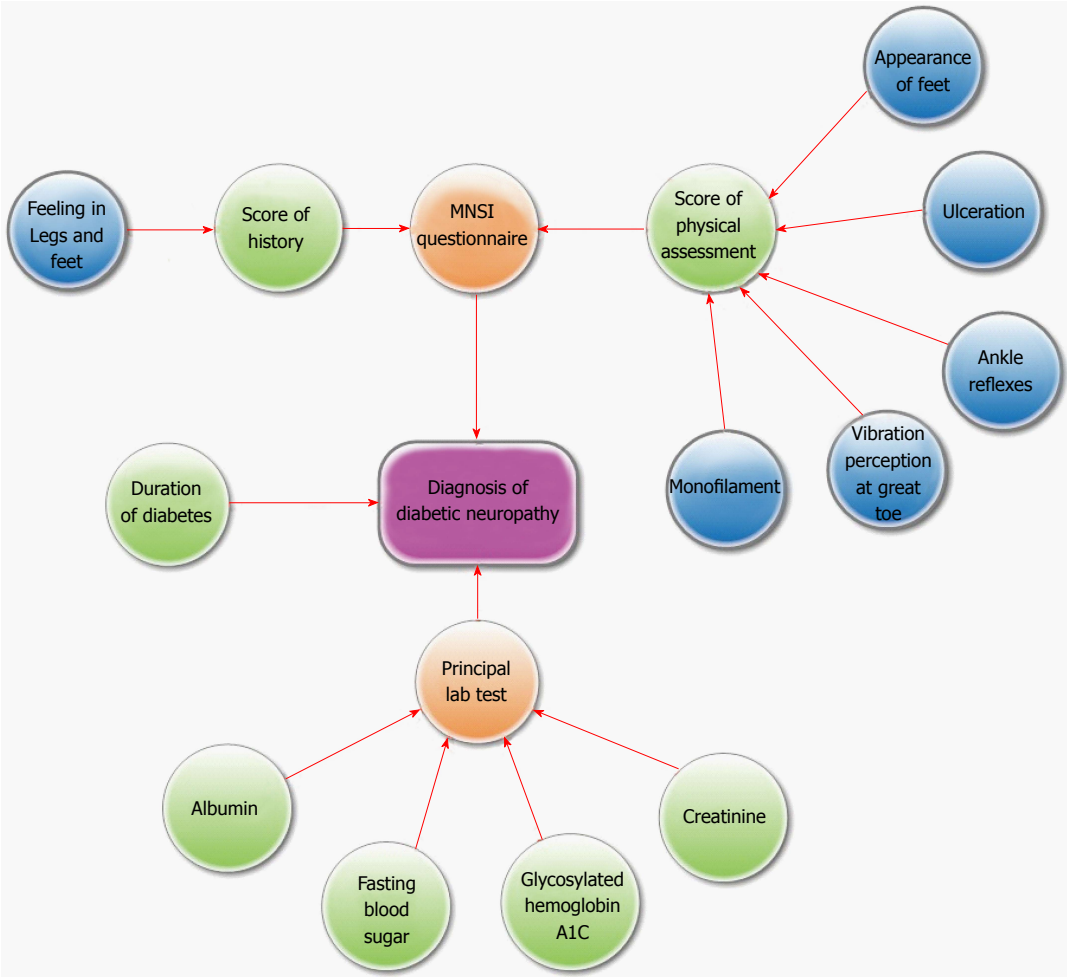

Figure 1 The semantic network of the expert system. MNSI: Michigan Neuropathy Screening Instrument.

an overview of the fuzzy inference architecture of the system.

Finally, the graphical user interface of the expert system was designed by using Active Server Page. Network Enabled Technology (ASP.NET). It is an open-source server-side web application framework designed for web development to produce dynamic web pages (Figure 3). The input variables, such as the duration

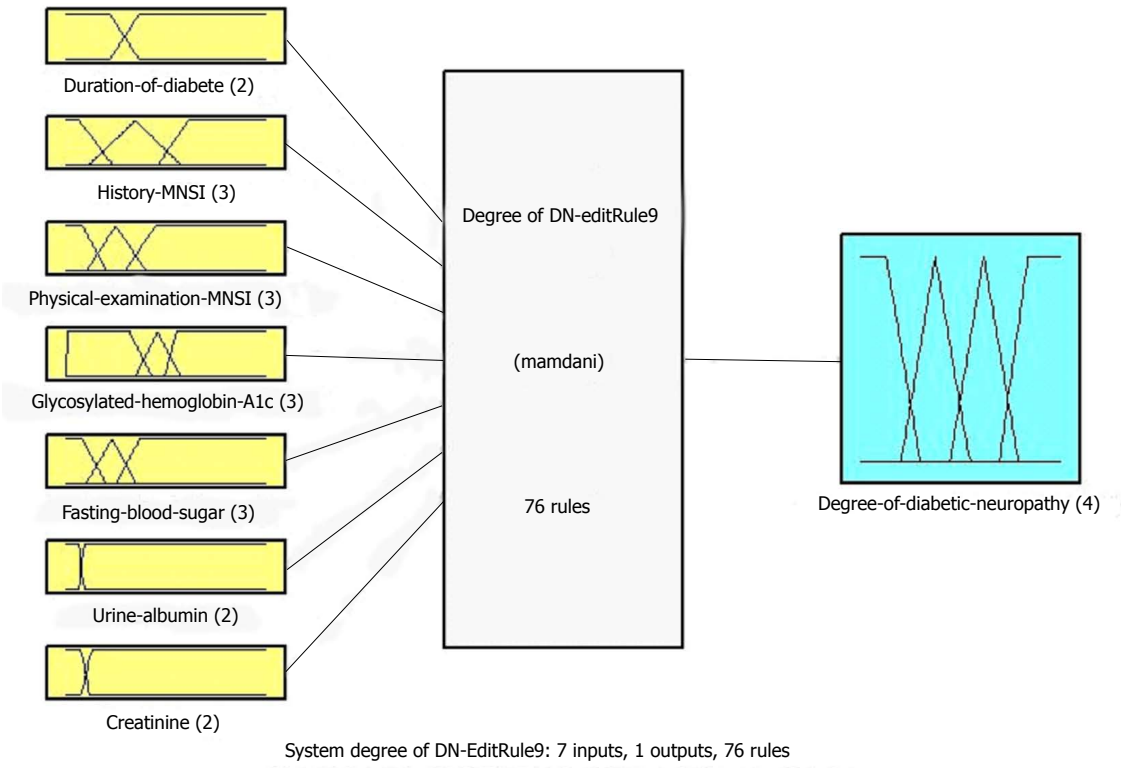

Figure 2 An overview of the fuzzy inference architecture of the system.

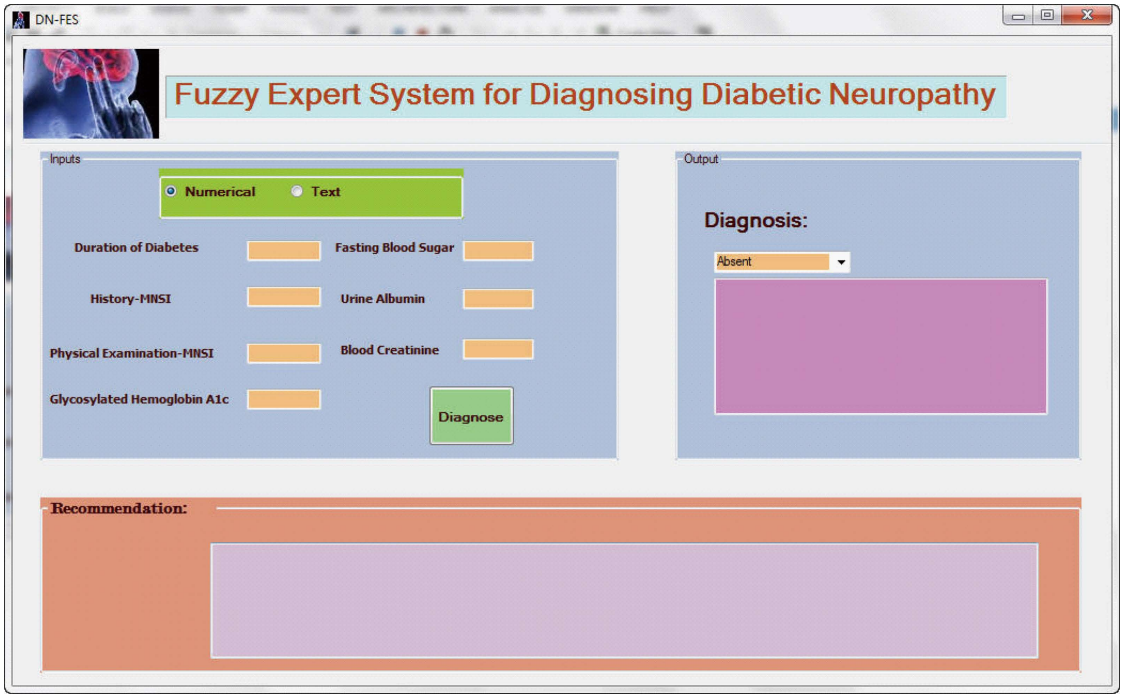

Figure 3 The graphical user interface of the fuzzy expert system.

of diabetes, the results of laboratory tests, and scores obtained from the Michigan questionnaire, could be entered into the system manually either in the textual or in the numerical format based on the user's choice. The output variable, namely the severity of the disease, which was shown as a number between zero and 10, had been divided into four categories: absence of the disease, (the degree of severity) mild, moderate, and severe. Figure 4 shows the risk of diabetic neuropathy based on the scores obtained from the Michigan questionnaire.

According to Figure 4, by increasing the scores

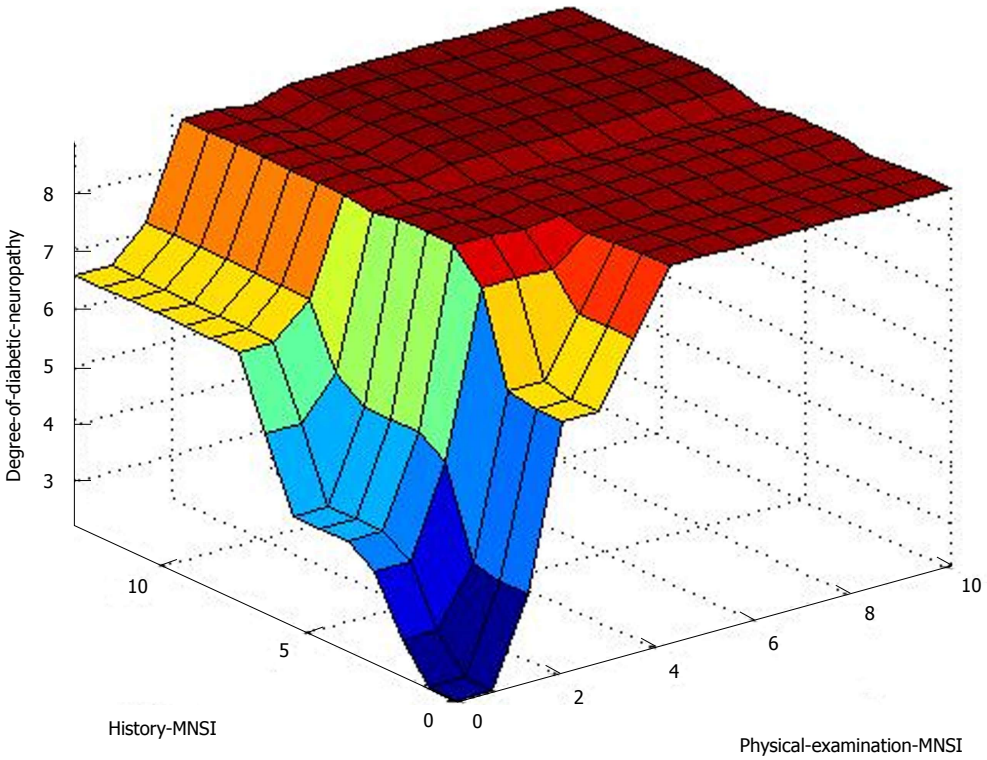

Figure 4 The risk of diabetic neuropathy based on the scores of the Michigan Neuropathy Screening Instrument questionnaire. MNSI: Michigan Neuropathy Screening Instrument.

obtained from the Michigan questionnaire, the severity of diabetic neuropathy will increase accordingly.

System function evaluation

The system was tested by using real data. In total, the records of 244 patients with diabetic neuropathy were identified. However, 31 records were excluded due to the incompleteness of clinical data. The remaining records (n = 213) included 118 patients who were diagnosed with diabetic neuropathy, while diagnosis was ruled out for the rest (n = 95). The system function was tested in terms of sensitivity (true positive rate), specificity (true negative rate), and accuracy (proportion of the true results, both positive and negative), which were 89%, 98%, and 93%, respectively.

Finally, the system's output was compared with the final diagnoses made by the specialists and recorded in the patients' records. These diagnoses were made by using the nerve conduction velocity test, the vibration perception threshold, the monofilament test, and the clinical neuropathy examination. The comparison was conducted by using the Kappa coefficient and the K value was 0.6. According to Landis and Koch, a Kappa value between 0.4 and 0.75 shows a fair to good agreement[25]. Therefore, the system designed in this study showed a fair to good level of similarity between the system's function and the specialists' diagnoses. The ROC curve presents the results of testing the system (Figure 5).

As can be seen in the above figure, the ROC curve is ideal. It is close to the high point of the square that represents an appropriate function of the system.

DISCUSSION

As mentioned before, one of the most common long-term complications of diabetes mellitus is diabetic neuropathy. In order to control this complication, it is important to diagnose it both accurately and timely[10]. Although there are a variety of methods to detect the disease, it is difficult to diagnose it at the very early stage[13]. Therefore, the use of IT applications, such as fuzzy expert systems, is suggested.

In the present study, seven diagnostic parameters-the duration of diabetes, the symptom assessment, the sign examination based on the MNSI, the glycolysis haemoglobin level, fasting blood sugar, blood creatinine, and albuminuria-were considered as input variables, and the severity of diabetic neuropathy was considered as an output variable. These variables were selected based on the specialists' perspectives and the literature review. Similarly, the knowledge and experience of four experts in the field of diabetic neuropathy was investigated in the study conducted by Picon *et al*[22] to determine the diagnostic parameters and to design a knowledge-based system. In their research, four inputs variables included symptom, the sign assessment based on the Michigan questionnaire, the glycolysis haemoglobin level, and the duration of diabetes. The output of the system classified the severity of diabetic neuropathy in three categories: Mild, moderate, and severe. In contrast with the study of Picon *et al*[22] the number of input variables increased

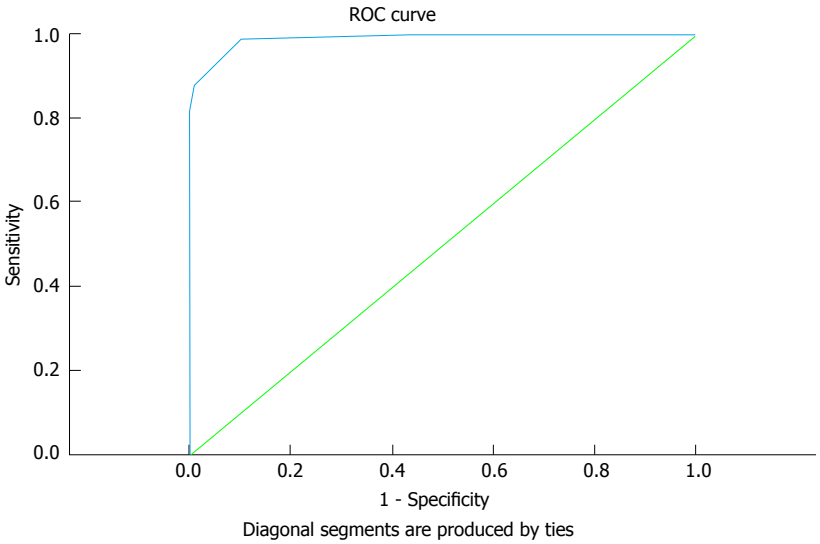

Figure 5 The receiver operating characteristic curve.

in the current research and laboratory test results were included to improve the accuracy of diagnosis. Similarly, Neshat *et al*[26]'s study considered six input variables and one output variable to diagnose liver disorders. To diagnose heart ailments, Adeli *et al*[27] used 12 input variables and considered the diagnosis of heart diseases as the output variable.

In the present study, values between zero and 10 were considered for the output variable, which was the severity of diabetic neuropathy. An increase in the value of output variable showed the level of severity for diabetic neuropathy.

In the current study, the fuzzy sets and membership functions for each of the seven input variables and the output variable were finalized after consulting a specialist. This approach can help eliminate the rules that could be covered by other rules, and finally, 76 rules were used to design the system. Similarly, DoostHoseini *et al*[28] consulted doctors to reduce the number of rules to an appropriate number. In another study, Zolnoori *et al*[29] developed a fuzzy expert system for diagnosing asthma. Given that the patients' records were incomplete, an indirect approach was used to develop the system's knowledge base. In this approach, the researchers reviewed books and scientific papers, and also conducted structured and unstructured interviews with doctors and patients. Having analysed the data, the most important variables useful for diagnosing asthma were identified.

In the present study, the system interface was designed by using ASP.NET rather than matrix laboratory (MATLAB). In fact, web-based applications have more flexibility and can be used by multiple users at the same time. Ease of use is another feature of these systems, which, in turn, can increase the work efficiency.

In this study, the output of the system was divided into four categories: The absence of the disease, mild, moderate, and severe. In contrast, Picon *et al*[22] classified the severity of neuropathy into three categories: Mild, moderate, and severe. Moreover, the specificity and sensitivity of the system were not reported in their study. In the current study, the specificity of the system was 98%, which shows a high level of system performance. Also, there was a relatively good agreement between the system's function and the diagnoses recorded by the specialists. Although other methods of diagnosis were not considered in the current study, the specificity and sensitivity of the system highly suggested that such a system could help physicians to diagnose the disease more quickly by using parameters like results of laboratory tests.

In the current study, the main aim was to develop an expert system for diagnosing diabetic neuropathy. Therefore, the clinical effectiveness of the system was not evaluated due to resource restrictions. Conducting evaluation studies after implementing the system in the actual healthcare setting would help determine the impact of the system on the health status of patients.

In conclusion, an expert system was designed for diagnosing diabetic neuropathy in this study. As diabetic neuropathy is a chronic disease that may have serious consequences, early diagnosis of the disease is important to control it. The system designed in the current study could help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. General practitioners can use such a system in remote areas to improve the quality of care for patients with diabetes. With it, the disease can be diagnosed more easily and quickly. There is no need to undertake complex procedures, and the care plan can be applied at the right time. Further research is suggested to increase the number of variables to improve the accuracy, sensitivity, and specificity of the system. Moreover, the feasibility of using this method in daily clinical practice and its impact on the efficiency and

cost-effectiveness compared to those of other methods need to be investigated in future studies.

COMMENTS

Background

One of the major problems associated with the diagnosis of diabetic neuropathy is the lack of reliable clinical scale for grading the severity of the disease. A variety of methods, such as the nerve conduction velocity test, the vibration perception threshold, and the monofilament test, are used to detect the peripheral neuropathy. In addition to clinical examination, laboratory tests and risk factors of the disease such as age, sex, renal disease, and smoking need to be considered.

Research frontiers

Since the disease usually develops on a continuous basis, two-valued logic cannot be used to express this continuity any more. Therefore, new methods for diagnosing the disease have been considered. Among these methods, the development of information technology applications, decision support systems, and fuzzy expert systems have received special attention.

Innovations and breakthroughs

In order to diagnose diabetic neuropathy, clinical examinations as well as results of laboratory tests like the haemoglobin A1c level, fasting blood sugar, and the oral glucose tolerance test should be considered. In this study, information technology was used to design a fuzzy expert system to diagnose the severity of diabetic neuropathy based on clinical examinations and laboratory tests.

Applications

The system designed in the current study can help specialists to diagnose the disease more quickly by using the most common diagnostic parameters. General practitioners, too, can use it in remote areas to improve the quality of care for patients with diabetes. With it, the disease can be diagnosed more easily and quickly. There is no need to undertake complex procedures, and the care plan can be applied at the right time.

Terminology

The fuzzy expert system is a new version of expert systems that uses fuzzy logic for data processing. A fuzzy expert system is used to describe uncertain phenomena because the real-world phenomena are much more complex than an exact and absolute description. The most common complication of diabetes is impairment of the peripheral neural system, which is known as diabetic neuropathy.

Peer-review

This is interesting and important paper for diagnosis of diabetic complications. The paper is well-written and focused.

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Contents

Monthly Volume 8 Number 3 March 15, 2017

MINIREVIEWS

ORIGINAL ARTICLE

Retrospective Cohort Study

Prospective Study

World Journal of Diabetes
Volume 8 Number 3 March 15, 2017

Contents

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MINIREVIEWS

Management of critically ill patients with diabetes

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Abstract

Disorders of glucose homeostasis, such as stress-induced hypoglycemia and hyperglycemia, are common complications in patients in the intensive care unit. Patients with preexisting diabetes mellitus (DM) are more susceptible to hyperglycemia, as well as a higher risk from glucose overcorrection, that may results in severe hypoglycemia. In critically ill patients with DM, it is recommended to maintain a blood glucose range between 140-180 mg/dL. In neurological patients and surgical patients, tighter glycemic control (*i.e.*, 110-140 mg/d) is recommended if hypoglycemia can be properly avoided. There is limited evidence that shows that critically ill diabetic patients with a glycosylated hemoglobin levels above 7% may benefit from looser glycemic control, in order to reduce the risk of hypoglycemia and significant glycemic variability.

Key words: Diabetes mellitus; Critical care; Stress hyperglycemia; Hypoglycemia; Glycemic control; Intensive care unit

Core tip: Diabetes mellitus is a common comorbidity found in critically ill patients. Although strict glycemic control in the past was considered a standard therapeutic intervention, newer clinical trials have shown that moderate glycemic control (*i.e.*, glucose levels between 140-180 mg/dL) reduces mortality and morbidity in such patients.

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INTRODUCTION

Stress-induced hyperglycemia, or diabetes injury as originally described by Claude Bernard in 1877, has become the subject of significant debate, as recent guidelines have called for stricter glucose control in critically ill patients[1,2]. Occurring as a result of catecholamine-induced stress response, this hyperglycemia is a common occurrence in critically ill patients[1]. With the rising population of diabetic and pre-diabetic individuals in the United States, the risk of severe hyperglycemia among critically ill patients is quite high, particularly in patients with undiagnosed diabetes mellitus (DM), who have inadequate glycemic control previous to hospitalization[1,3].

On the other hand, one of the important complications in dealing with stress-induced hyperglycemia is severe hypoglycemia. This significant decrease in blood glucose, however, is not due to some underlying physiological process, but it is often the consequence of inadequate glucose monitoring, and incorrect dosage of hypoglycemic medication, usually insulin. Hypoglycemia in critically ill patients is an important factor that can increase mortality in the intensive care unit (ICU), and is an important complication that needs to be prevented in patients that require glycemic control therapy[4]. Increased glycemic variability may be an issue with inadequate hypoglycemic treatment, which leads to increased oxidative stress and may be more dangerous than persistent hyperglycemia[5].

Appropriate hypoglycemic therapy is required in order to reduce mortality and morbidity of uncontrolled hyperglycemia in critically ill patients[6]. In this article, we review the current state-of-evidence on ideal glycemic goals that should be set for diabetic patients in the ICU.

EPIDEMIOLOGY

In 2014, the United States National Diabetes Statistic Report, documented 21 million individuals suffering from DM, accounting for 6.7% of the total population and approximately 8.1 million undiagnosed DM, which would raise the percentage of American population with diabetes to 9.3%[7]. This report also indicated that the prevalence of diabetes was highest among those older than 65 years of age and above[7]. Patients in this age group, account for up to 45.7% of ICU patients[8]. In addition, approximately 50% of ICU patients, have pre-existing diagnostic criteria for DM[9].

PATHOPHYSIOLOGY

During periods of stress, the body reacts by producing elevated levels of catecholamines[10]. This reaction, is modulated by the suprarenal glands and activated by either the sympathetic nervous system in acute stress and by feedback to the pituitary gland in chronic stress[11,12]. Any period of disease can be considered a period of stress, and therefore, some degree of hyperglycemia is normal during these times, and can be seen as initially protective and part of the adaptive response for survival[13]. However, in acute and severe diseases, the resulting hyperglycemia can be much too high and require glycemic control therapy to manage[1].

Severe hyperglycemia, is a well-documented marker of illness severity, rather than a direct cause of poor outcome[13]. This condition often subsides after the affecting illness (*i.e.*, sepsis) has resolved[1]. In the acute setting, it is believed that the resulting hyperglycemia is due to insufficient insulin secretion that is unable to overcome the hyperglycemic effect of catecholamine.[14] It is also believed that insulin resistance plays a factor in chronic disease with significant amounts of tissue injury[1,14].

Patients with pre-existing DM tend to have a persistent state of hyperglycemia due to insulin resistance (or insulin absence in DM type 1), and hyperglucagonaemia that are the consequences of the disease's natural progression. As a result of these factors, during periods of acute illness, the resulting stress-induced hyperglycemia can be much more severe than in non-diabetic patients, and more likely to require control with hypoglycemic medications and strict glucose monitoring[14]. See Table 1 for factors that lead to hyperglycemia and hypoglycemia in critically ill patients.

STRESS-INDUCED HYPERGLYCEMIA

Stress-induced hyperglycemia (SIH) is a common finding among critically ill patients, particularly among cardiovascular patients, neurocritical patients, and patients undergoing surgical procedures, even in the absence of preexisting DM[14]. In non-diabetic patients, SIH has been arbitrarily defined as a blood glucose level greater than 140 mg/dL or glycosylated hemoglobin (HbA1c) greater than 6.5%[15]. In diabetic patients, SIH is be defined as blood glucose levels greater than 180-220 mg/dL[15]. This clinical condition increases the morbidity and mortality in critically ill patients and leads to poor outcomes and prognosis[15]. Some have advocated that in these patients, it is necessary to maintain a strict glycemic control to directly improve their outcomes[14,15].

Part of the controversy as to the precise level of strict glycemic control started with a clinical study published in 2001, consisting of 1548 patients in a surgical ICU in Belgium[16]. In this study, van den Berghe *et al*[16] reported that intensive insulin therapy, aimed at maintaining blood glucose below 110 mg/dL reduced mortality and morbidity in critically ill patients by 42%. The reduction in mortality was apparent among patients who stayed in the ICU for more than five days[16]. A follow-up study, by the same investigators in 2006, aimed at comparing strict blood glycemic control (blood glucose: 80-110 mg/dL) *vs* a much looser control (blood glucose: 180-215 mg/dL) in this study on 1200 medical

Table 1 Factors leading to hyperglycemia and hypoglycemia in critically ill patients

Hyperglycemia[65]	Hypoglycemia[66]
Release of stress hormones (glucagon, epinephrine, cortisol, and TNF-α)	Severe sepsis
Certain medications (exogenous glucocorticoids, vasopressors, lithium, and β-blockers)	Trauma
Overfeeding	DM
Intravenous dextrose	Prior insulin treatment
Parenteral nutrition	Prior glucocorticoid treatment
Persistent bed rest	Cardiovascular failure
Increased insulin resistance (DM type 2)	Intensive glucose control
Deficient insulin secretion (DM type 1)	

DM: Diabetes mellitus; TNF: Tumor necrosis factor.

ICU patients and found that the strict glucose control group had a mortality reduction rate of 32% in patients who stayed more than three days in the ICU[17]. Of note, in this study, strict glucose control increased mortality in patients with short ICU stays (< 3 d), due to the increased rate of severe hypoglycemia.

A series of additional clinical trials followed these 2 seminal investigations. One of the most quoted in the medical literature was the Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) multicenter trial, with 6104 ICU patients that compared strict glucose control (81-108 mg/dL) *vs* a more moderate glucose target (< 180 mg/dL)[18]. This study reported that moderate glycemic control lead to a reduction in cardiovascular mortality in critically ill patients.

Glycemic variability and hypoglycemia

As noted above, in diabetic patients, particularly those with persistent hyperglycemia, significantly lowering glucose levels and strict glycemic control may lead to symptomatic and life-threatening hypoglycemia and glycemic variability[19]. Glycemic variability has been defined as acute glycemic fluctuations; with both upwards fluctuations (in hypoglycemic correction) and downward fluctuations (in initial overbearing hypoglycemic treatment) leading to increased oxidative stress (which in turn leads to endothelial dysfunction and vascular damage). It is well documented that glycemic variability is much more dangerous than persistent hyperglycemia in critically ill patients[19,20].

Two retrospective studies found that glycemic variability conferred an increased risk of mortality in critically ill patients[21,22]. The mortality risk increased by 25.7% in critically ill non-diabetic patients[21,22]. Although no current consensus exists on the adequate range of acceptable glycemic variability in critically ill patients, Monnier and associates proposed a range of 40 mg/dL, as this corresponds to the normal variability found in non-diabetic healthy individuals[20].

Hypoglycemia is another dangerous situation in both diabetic and non-diabetic ICU patients. This clinical entity is directly related to cardiovascular mortality as it has been associated with increased QT waves in the electrocardiogram and changes in cardiac cell repolarization[23,24]. A study performed in 2005 reported that diabetic patients hospitalized with acute myocardial infarction, had a 93% increased mortality rate when hypoglycemia was present during their hospitalization[25]. In another study published last year, 2601 patients were evaluated and analyzed ICU mortality when moderate or severe hypoglycemia was present as compared to no hypoglycemia. Patients with severe and moderate hypoglycemia had a 34% and 18% increase, respectively, in ninty days mortality, when compared to patients with no hypoglycemia. Those patients that presented multiple hypoglycemic events had a 44% increase in mortality when compared to patients with no hypoglycemic events[26].

There is significant evidence that hypoglycemia poses significant risk of cardiovascular mortality among diabetic patients in critical care scenarios. Alongside the theoretical benefits of reducing glycemic variability, having a much looser glycemic control in critically ill diabetic patients, may aid in reducing cardiovascular mortality[27]. Further studies are necessary on the subject of glycemic variability, in an effort to find its real-world impact on diabetic patients in and out of critical care.

GUIDELINES RECOMMENDATIONS

The American Diabetes Association recommends starting insulin in patients with persistent hyperglycemia above 180 mg/dL in critically ill patients, and to maintain the glycemic range between 140-180 mg/dL. It also states that stricter glycemic control (110-140 mg/dL) can be appropriate for certain patients, such as patients with acute cardiac ischemia or patients with acute neurological event, as long as significant hypoglycemia can be avoided[28]. They also recommend active prevention of hypoglycemia by having a treatment plan if hypoglycemia were to develop and to change the current therapy if serum glucose levels fall below 70 mg/dL[28]. These recommendations were based on a consensus form American Association of Clinical Endocrinologists, which involved two meta-analyses of several clinical trials, including the NICE-SUGAR study, the largest randomized controlled trial, addressing this issue[28-31].

The American College of Physicians recommends serum glucose levels between 140-200 mg/dL indepen-

dent of diabetic status, and recommends avoiding blood sugar levels below 140 mg/d, due to the associated risks of hypoglycemia and glycemic variability[32]. The Society of Critical Care Medicine (SCCM) recommends maintaining the serum glucose level between 150-180 mg/dL[33].

However, a 2011 study conducted in the ICU among diabetic patients found that patients with uncontrolled diabetes (HbA1c above 7%) had different mortality when hyperglycemia was present when compared to non-diabetic patients or patients with better controlled diabetes (HbA1c below 7%)[34]. Additional newer studies have concluded similarly, that diabetic patients do not share the same mortality with hyperglycemia as non-diabetic patients, and that these diabetic patients may benefit from higher glycemic ranges to reduce the risk of hypoglycemia and glycemic variability[35-37]. Moreover, another study recommended maintaining serum glucose levels between 160-220 mg/dL in patients with HbA1c above 7%, and to maintain serum glucose levels between 140-200 mg/dL in patients with an HbA1c below 7%[19].

It is recommended that glycemic control be maintained with insulin due to the effectiveness, quick action, and few contraindications as it relates to this therapy[28,29]. However, the use of continuing metformin therapy in ICU patients with type 2 diabetes is seeing resurgence among certain patients, as the risk of hypoglycemia is lower; although its use should be cautious among patient with renal insufficiency, which is very common in the ICU[38].

In the following sections, we describe the evidence and recommendations for glycemic control among different patient groups who may be presenting in the ICU. Details are depicted in Table 2.

Patients in the surgical ICU

The Society of Thoracic Surgeons created guidelines in 2009 for glucose management in adult cardiac surgery patients, including diabetics[39]. For preoperative care, maintenance with insulin therapy with a serum glucose goal below 180 mg/dL was recommended. It was also recommended to check HbA1c level pre-operative for proper glycemic management. Intraopertavely, insulin therapy was also recommended for glycemic values above 180 mg/dL, and intravenous insulin infusion was recommended for persistent glycemic levels above 180 mg/dL intra-operatively or postoperatively in the ICU[39]. The recommendation was to keep a goal of 180 mg/dL throughout their stay in the ICU unless they are expected to remain in the critical care unit more than 3 d, or if the patient is ventilator-dependent, or requires therapy with inotropes, intra-aortic balloon pump, left ventricular assist device, anti-dysrhythmic medications, dialysis, or hemofiltration. In aforementioned cases, it is recommended to have the blood glucose levels below 150 mg/dL[39,40].

A recent study in 447 patients, found that a glucose level of 80-110 mg/dL, when compared to 140-180 mg/dL, reduces surgical site infections[41]. However, this study did not focus on over-all patient mortality and had a challenge of small sample size.

Patients with neurological events

A large clinical trial by van den Berghe *et al*[16] in 2001, suggested that strict glucose control (< 110 mg/dL) reduces mortality in critically ill patients[16]. For a period of time, following the findings of this trial, the standard of care was to maintain neurocritically ill patients blood glucose below 110 mg/dL[16]. However, the publication of the NICE-SUGAR study, and a prospective study of intensive insulin therapy in patients with recent neurosurgery, both published in 2009, showed that strict glucose control led to increased mortality mainly secondary to hypoglycemia[18,42].

In 2012, a systematic review and meta-analysis of 16 clinical trials on optimal glycemic control in neurocritical care patients, revealed that strict glycemic control (70-140 mg/dL) had no impact on patient mortality, but did increased the incidence of hypoglycemia[43]. Loose glycemic control (> 200 mg/dL) was shown to increased mortality when compared to a moderate glycemic control (140-180 mg/dL)[43]. The ADA states that blood glucose level of 110-140 mg/dL may be appropriate if significant hypoglycemia can be avoided[28].

Patient with an acute myocardial infarction

In 2008, the American Heart Association released a statement on glucose management in acute coronary syndrome, which recommended a glucose levels between 90-140 mg/dL in ICU patients with acute coronary syndrome[44]. The recommendations were later updated in 2009, suggesting an upper limit of serum glucose to 180 mg/dL[45].

The European Society of Cardiology published their most recent guidelines in 2012 on management of acute myocardial infarction with ST-segment elevation[46]. They recommend loose glycemic control in the acute phase, by maintaining the patient serum glucose below 200 mg/dL, as hypoglycemia was felt to be an important factor which increases the mortality[46]. This conclusion is based on a consensus reached by the National Institute Health and Care Excellence in 2011, that stated that no high quality studies were available to reach an evidence-based conclusion[47].

A 2012 meta-analysis, focusing on type 2 diabetics with acute myocardial infarction, involving 3 studies (for a total of 2113 patients), concluded that stricter glucose control with intensive insulin therapy did not reduced the patient mortality but significantly increased the incidence of hypoglycemia while offering no overall reduction in cardiovascular mortality[48].

Patients with sepsis

In response to the study on glucose control in surgical ICU patients, a study specifically on patients with sepsis, the Surviving Sepsis Campaign recommended a

Table 2 Glycemic control recommendation based on patient condition

Condition	Glucose control recommendation	Studies with patient number	Ref.
Non-diabetic ICU patients	140-180 mg/dL	29 studies with 8432 total patients and 26 studies with 13567 total patients	Wiener *et al*[30] (2008) and Griesdale *et al*[31] (2009), respectively
Diabetic ICU patients	If HbA1c < 7%: 140-180 mg/dL If HbA1c > 7%: > 200 mg/dL	1 retrospective study with 415 total patients	Egi *et al*[34] (2011)
Surgical ICU	If ICU stay is for more than 3 d, ventilator dependent, on dialysis, or with cardiac comorbidities: < 150 mg/dL	1 prospective study with 4864 total patients across 17 yr	Furnary *et al*[40] (2004)
Neurocritical ICU patients	If not: < 180 mg/dL If hypoglycemia can be prevented: 110-140 mg/dL If not: 140-180 mg/dL	16 studies with 1258 total patients	Kramer *et al*[43] (2012)
STEMI ICU patients	< 200 mg/dL	No high quality studies available Consensus by NICE	Nice Guidelines[47] (2011)
Sepsis ICU patients	< 180 mg/dL	1 randomized control trial with 6104 patients	Based of NICE-SUGAR study[17]
Pregnant ICU patients	No consensus	N/A	Van de Velde *et al*[55] (2013)

ICU: Intensive care unit; N/A: Not applicable; HbA1c: Glycosylated hemoglobin; NICE-SUGAR: Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation.

stricter range of glycemic control, with an upper goal of 110 mg/dL of serum glucose[17,49,50]. With the advent of the NICE-SUGAR trial in 2009, which also included septic patients, the 2013 update of the Surviving Sepsis Campaign modified its recommendation to a looser goal of 180 mg/dL[51]. Due to increased risk of hypoglycemia and hypoglycemia-related mortality, the Surviving Sepsis Campaign deemed that there was no apparent benefit from strict glucose control[51]. Insulin therapy was recommended to be started after two consecutive blood glucose measurements were above 180 mg/dL and to maintain a blood glucose of less than 180 mg/dL[51].

Pregnant patients

Gestational diabetes accounts for 2% to 9% of all pregnancies[52]. Hyperglycemia is an important factor to consider in all pregnancies, especially among hospitalized patients. During pregnancy, maternal cells have increased insulin resistance, due to elevated levels of human placental lactogen, progesterone, and estrogen[53]. This mild increase in insulin resistance is protective, and allows glucose absorption to be prioritized in the fetus, however in some patients, this mild resistance can be combined with insulin resistance, leading to persistent hyperglycemia[53,54].

It is generally agreed that treatment of gestational diabetes-related hyperglycemia is important in reducing perinatal mortality, as well as reducing hyperglycemia in postpartum mothers and improving overall health[52]. No consensus currently exists on the ideal range of serum glucose levels in critically ill pregnant patients[55]. It is difficult to recommend moderate or loose glycemic control in these patients, as even mild hyperglycemia can lead to adverse outcomes in infants[56]. On the other hand, tight glycemic control may lead to increased risk of hypoglycemia, which is also a factor that increases both maternal and infant mortality. Future clinical trials are necessary to be able to reach a consensus on how glycemic care should be managed in this population.

GLYCEMIC CONTROL THERAPY

While several studies have been performed on glycemic control in non-diabetic patients in the ICU, few of such studies have been performed on diabetic individuals. Table 3 depicts recent studies on this topic. Three of the four studies focused on surgical patients, and recommend a stricter glucose control for infection prevention, and hyperglycemia prevention[57-59]. The fourth study takes into account the risk of hypoglycemia, and recommends looser glycemic control to reduce moderate to severe hypoglycemia and glycemic variability[9]. However, all of these studies fail to take into account that diabetic individuals with persistent hyperglycemia (HbA1c above 7%) who are at higher risk from hypoglycemia-related mortality than hyperglycemia-related mortality[19,34]. A 2016 study on diabetic ICU patients, recommended keeping serum glucose levels below 250 mg/dL in patients with HbA1c above 7% upon admission to the ICU[9]. This study found that this loose glycemic control prevented glycemic variability and reduced the incidence of moderate and severe hypoglycemia[9].

Measurement of glucose should be performed every 2 to 4 h to allow for proper monitoring. If the patient's serum glucose concentration is fluctuating, it may be necessary to measure glucose every 30 or 60 min[60]. Currently, technology for continuous blood glucose monitoring using vascular catheter blood sampling is currently undergoing clinical trials and may become the standard of care and can allow tighter glycemic control in addition to preventing severe hypoglycemia or hyperglycemia[61]. Research has shown promise, as the technology is capable of detecting changes in glycemia that may otherwise be missed in our current practice, and has

Table 3 Strict glycemic control *vs* moderate glycemic control in critically ill patients with diabetes

Ref.	Study design/cohort	Sample size	Control group	Therapies employed	Conclusion	Favored therapy
Lecomte *et al*[57] (2011)	Diabetics undergoing off-pump cardiac bypass surgery	60	Matched 60 non-diabetics	Strict glycemic control (80-110 mg/dL)	Strict glycemic control was feasible and efficient Minimal risks for hypo- or hyperglycemia	Strict glycemic control
Yuan *et al*[58] (2015)	Diabetic patients receiving enteral nutrition after gastrectomy	212	None	Strict glycemic control (80-110 mg/dL) and moderate glycemic control (< 200 mg/dL)	Strict glycemic control lead to higher rates of severe hypoglycemia but lower rates of severe hyperglycemia Surgical site infection rate was higher with moderate glycemic control Rates of other complications were similar in the two groups	Strict glycemic control
Umpierrez *et al*[59] (2015)	Diabetic patients after coronary artery bypass surgery	152	150 non-diabetics	Strict glycemic control (100-140 mg/dL) and moderate glycemic control (141-180 mg/dL)	No significant differences between the two in the rate and severity of complications	Neither
Kar *et al*[9] (2016)	Diabetic ICU patients with HbA1c ⩾ 7.0% admission	83	None	Moderate glycemic control (< 180 mg/dL) and Loose glycemic control (< 250 mg/dL)	Loose glycemic control reduces glycemic variability and moderate to severe hypoglycemia	Loose glycemic control

ICU: Intensive care unit; HbA1c: Glycosylated hemoglobin.

shown that glucose levels correlate well with standard arterial glycemic measurement[62-64].

CONCLUSION

Glycemic control in the ICU continues to be challenging at best. Although the glycemic control strategy does not vary among diabetic individuals without persistent hyperglycemia from non-diabetic individuals (serum glucose goal of 140-180 mg/dL), it is important to note the cases where exceptions should be made. In neurological patients and surgical patients, a stricter glycemic strategy can be maintained (110-140 mg/dL and < 150 mg/dL, respectively) as long as adequate hypoglycemia can be avoided. In patients with a history of persistent hyperglycemia (HbA1c above 7%), liberal glycemic control may be beneficial in reducing the risk of hypoglycemia and glycemic variability, which is known to increase cardiovascular mortality, but further evidence and studies are necessary before a strong recommendation can be given. Further randomized control studies are suggested to further evaluate the variability in the target blood glucose level among different conditions.

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MINIREVIEWS

Involvement of Cbl-b-mediated macrophage inactivation in insulin resistance

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Abstract

Aging and overnutrition cause obesity in rodents and humans. It is well-known that obesity causes various diseases by producing insulin resistance (IR). Macrophages infiltrate the adipose tissue (AT) of obese individuals and cause chronic low-level inflammation associated with IR. Macrophage infiltration is regulated by the chemokines that are released from hypertrophied adipocytes and the immune cells in AT. Saturated fatty acids are recognized by toll-like receptor 4 (TLR4) and induce inflammatory responses in AT macrophages (ATMs). The inflammatory cytokines that are released from activated ATMs promote IR in peripheral organs, such as the liver, skeletal muscle and AT. Therefore, ATM activation is a therapeutic target for IR in obesity. The ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) appears to potently suppress macrophage migration and activation. Cbl-b is highly expressed in leukocytes and negatively regulates signals associated with migration and activation. Cbl-b deficiency enhances ATM accumulation and IR in aging- and diet-induced obese mice. Cbl-b inhibits migration-related signals and SFA-induced TLR4 signaling in ATMs. Thus, targeting Cbl-b may be a potential therapeutic strategy to reduce the IR induced by ATM activation. In this review, we summarize the regulatory functions of Cbl-b in ATMs.

Key words: Casitas b-lineage lymphoma-b; Insulin resistance; Macrophage; Obesity; Toll-like receptor 4

Core tip: Obesity leads to the development of chronic inflammation and insulin resistance (IR). Adipose tissue macrophages (ATMs) play a crucial role in the development of obesity-induced IR. Therefore, ATMs are attractive therapeutic targets for IR. Recently, we demonstrated that the ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) negatively regulates the migration and activation of ATMs.

Here, we review key aspects of Cbl-b function in the regulation of ATMs.

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INTRODUCTION

In 2014, more than 1.9 billion adults were overweight, and of these, over 600 million were obese[1]. Obesity is a risk factor for the development of insulin resistance (IR), diabetes mellitus, hepatic steatosis and hypertension[2], resulting in escalating healthcare costs in several developed countries. Thus, it is important to elucidate the mechanism for obesity-associated IR and develop attractive therapeutic strategies for treating IR. A combination of various factors, such as diet, lifestyle, genetic background, psychological stress and aging, leads to obesity. In particular, aging and nutritional excess play critical roles in the development of obesity.

Aging causes decreases in physical activity, lean body mass and anti-oxidant defenses, thus increasing oxidative stress and the number of damaged cells[3]. These changes are associated with lipid accumulation in white adipose tissue (WAT) due to decreased energy expenditure. The oxidative stress induced by aging causes mitochondrial dysfunction and muscle atrophy. Sarcopenia, aging-induced skeletal muscle loss, decreases energy expenditures and causes obesity[4]. An excessive intake of carbohydrates and lipids causes the accumulation of triacylglycerols in adipocytes, which produces expansion of the adipocyte. Obesity causes inflammatory responses in WAT. It is well-known that in addition to its roles in fat storage, AT also plays key roles in endocrine system. AT secretes lipids, adipokines and chemokines to maintain homeostasis. The hypertrophy of the AT alters adipokine and chemokine secretion[2,5]. It is well-known that diverse immune cells reside in WAT of both lean and obese individuals, and these cells release inflammatory cytokines during obesity. Resident eosinophils and regulatory $CD4^+$ helper T cells maintain homeostasis in the AT of lean subjects[6]. In contrast to $CD4^+$ T cells, $CD8^+$ T cells increase in number in the AT of obese subjects and promote the inflammatory responses mediated by macrophages[7]. AT macrophages (ATMs) also release various inflammatory mediators. Because ATMs play a key role in obesity-associated inflammatory action, the suppression of ATM activation is a potent therapeutic strategy for treating IR induced by obesity. Recently, several studies demonstrated that the ubiquitin ligase Casitas b-lineage lymphoma-b (Cbl-b) is a key regulator of macrophage activation[8-10]. Here, we review the key roles of Cbl-b in ATM activation and the pathogenesis of IR in obesity.

THE UBIQUITIN LIGASE CBL-B

In mammalian cells, there are three major intracellular protein degradation pathways. The calpain pathway, the autophagy-lysosome pathway, and the ubiquitin (Ub)-proteasome system play important roles in maintaining cellular homeostasis. In particular, the Ub-proteasome system is regulated by three types of enzymes: A Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub ligase (E3). In the initial step, the activation of Ub proteins by E1 enzymes is critically dependent on the presence of ATP. An E1 enzyme transfers a Ub protein to E2 enzyme. And then, the E2 enzymes shuttle a Ub protein to an E3 enzyme, which ubiquitinates the specific target protein. The proteins tagged with Ub are specifically degraded by the proteasome. Therefore, E3 enzymes are important for determining the specific target proteins that will be degraded by proteasome[11].

The Cbl proteins in mammalian (c-Cbl, Cbl-b and Cbl-c), which were originally identified as adaptor molecules, function as ubiquitin ligases (Figure 1). A number of studies show that Cbl proteins inhibit the signal transduction by receptor and non-receptor tyrosine kinases[12-14]. The protein tyrosine kinase-binding (TKB) and really interesting new gene (RING) finger (RF) domains are highly conserved in the N-terminal domains of all Cbl homologues. The TKB domain, which is a specific domain in Cbl proteins, binds to the phosphorylated tyrosines of the substrates through Src-homology (SH) 2 domains[15]. The RF catalytic domain has the E3 ubiquitin ligase activity because it binds to E2 enzymes[16]. Cbl-b is a substrate of tyrosine kinases, and the ubiquitin ligase activity is regulated by the phosphorylation of some tyrosine residues[14,17,18]. Increasing evidence indicates that Cbl-b is abundantly expressed in leukocytes and decreases the activation of various immune cells. Therefore, loss-of-function mutations of *Cblb* cause various autoimmune diseases[19-21]. Interestingly, a *Cblb* mutation was identified as factor associated with diabetes in a rat model of human type Ⅰ diabetes[20,22]. Yokoi *et al*[22] reported that F328L is a loss-of-function mutation in T cells that was identified in Japanese subjects. These studies reveal that the function of Cbl-b is connected to diabetes.

INFLAMMATORY ACTIONS OF MACROPHAGES IN ADIPOSE TISSUE

Various immune cells, such as macrophages, T cells, mast cells, natural killer cells and eosinophils, reside in WAT along with adipocytes. The expansion of adipocytes alters these populations in WAT. ATMs increase the number of cells in the AT of obese mice[23]. ATMs play important roles in the AT of lean and obese humans and rodents. In the AT of lean subjects, resident M2-like or alternatively activated ATMs preferentially maintain homeostasis by secreting anti-inflammatory cytokines. In contrast, in obesity, the M1-like or classically activated ATMs in WAT induce inflammation mediated by the release of inflammatory cytokines and

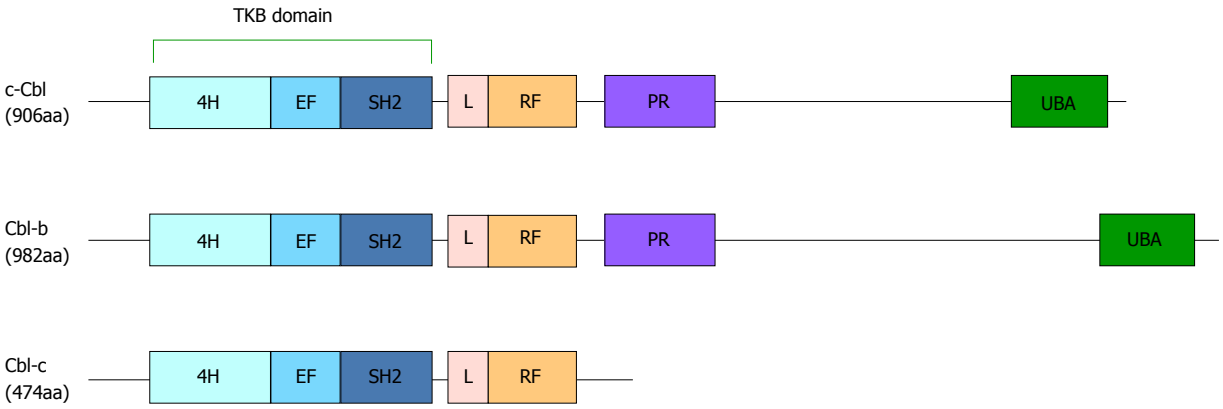

Figure 1 The primary structure and domain organization of human Casitas b-lineage lymphoma family proteins. Cbl-b proteins contain highly conserved tyrosine kinase-binding (TKB), linker (L), RING finger (RF) and proline-rich (PR) domains. 4H: Four-helix bundle; SH2: Src-homology 2; UBA: Ubiquitin-associated domain.

chemokines. ATMs are activated by saturated fatty acids (SFAs) through toll-like receptor 4 (TLR4). Although TLR4 was identified as the receptor for lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative bacteria[24], SFAs also activate TLR4 signaling in macrophages. The global mutation or the bone marrow-specific deficiency of TLR4 abrogated the systemic IR induced by the consumption of a high-fat diet (HFD)[25-27]. However, the molecular mechanism of TLR4 activation by SFAs is poorly understood. It is thought that SFAs fail to directly bind to TLR4[28]. A recent study[29] showed that SFAs activate the TLR4 signaling mediated by fetuin-A, a 64 kDa glycoprotein released from the liver in response to HFD consumption. Fetuin-A mediates SFA-induced activation of TLR4 by directly interacting with TLR4 in macrophages and adipocytes[29]. Interestingly, treatment with the insulin sensitizer pioglitazone suppresses fetuin-A expression through peroxisome proliferator-activated receptor-γ activation in hepatoma cells[30]. SFA treatments induce the activation of nuclear factor κB (NF-κB) and Jun N-terminal kinase (JNK), which are TLR4 signaling molecules in macrophages[26,31]. In fact, the inhibition of NF-κB or JNK ameliorates IR by activating ATMs in obese rodents[32,33]. Therefore, the regulation of ATM activation is a potent therapeutic target for obesity-associated IR.

CBL-B IN ATM RECRUITMENT

Aging and overnutrition cause the hypertrophy of AT, resulting in the accumulation of ATMs[5]. The activated ATMs induce peripheral and systemic IR through the release of inflammatory cytokines. JNK is a TLR4 signaling molecule and mediates the expression of inflammatory cytokines in macrophages. Bone marrow-specific deficiency of JNK1 ameliorated diet-induced IR by suppressing AT inflammation in mice[34]. We demonstrated that depletion of Cbl-b exacerbated obesity and IR induced by aging and HFD in mice[35,36]. We also found that ATM activation was enhanced in Cbl-b knockout (Cbl-b$^{-/-}$) mice. In 30-wk old Cbl-b$^{-/-}$ mice, we observed hypertrophy of AT, IR, hepatic steatosis and β cell dysfunction (Table 1). Interestingly, the ATM accumulation was dramatically increased in WAT. This event was caused by two factors in Cbl-b$^{-/-}$ mice. One factor was the high levels of monocyte chemotactic protein (MCP)-1/CC chemokine ligand 2 protein in circulation and WAT. MCP-1 is a member of CC chemokines, and causes the chemotaxis of leukocytes[37]. Previous reports demonstrated that MCP-1 and CC chemokine receptor type 2 (CCR2), the receptor for MCP-1, are associated with obesity-induced IR, inflammation and ATM accumulation[38-41]. In addition, CCR2 causes hepatic infiltration of macrophages and steatosis in mice[42,43]. Taken together, the data indicate that the inhibition of CCR2 is a potent therapeutic strategy for treating obesity-induced inflammation and IR.

Furthermore, it is known that Cbl-b decreases the migration-related signaling in macrophages. Macrophage migration is regulated by activation of the guanine nucleotide exchange factor Vav1[44]. Previous studies demonstrated that phosphorylation of Vav1 at Tyr267 mediated by spleen tyrosine kinase (Syk) is critical for Vav1 activation in leukocytes[45,46]. Cbl-b directly binds to Vav1 in T cells[47,48]. Although Vav1 phosphorylation is inhibited by Cbl-b, Cbl-b does not induce the degradation of Vav1. We also demonstrated that the depletion of Cbl-b promoted tyrosine phosphorylation in Vav1 in peritoneal macrophages from mice. These results indicated that the increased MCP-1 released from WAT and Vav1 phosphorylation cause ATM accumulation in Cbl-b$^{-/-}$ mice (Figure 2). In fact, treatment with an anti-MCP-1 antibody reduced the IR and ATM accumulation in Cbl-b$^{-/-}$ mice. Thus, Cbl-b may serve as a therapeutic target to reduce the IR mediated by the accumulation of ATMs.

CBL-B IN TLR4 SIGNALING

Several ubiquitin ligases have been identified as negative regulators of TLR4 signaling[49-52]. Triad3A is a RF ubiquitin ligase and directly binds to TLR4, resulting in ubiquitination and proteolytic degradation. Recent reports indicate that TLR4 signaling is inhibited by Cbl-b in macrophages and neutrophils[8,53]. Han *et al*[8] demonstrated that TLR4 signaling induced by LPS was suppressed in macrophages

Table 1 Phenotypes of Cbl-b$^{-/-}$ mice

Age and diet	Phenotypes	Ref.
30-wk old, normal diet	Adipose tissue inflammation Adiposity Fasting hyperinsulinemia Hepatic steatosis Impaired glucose tolerance Insulin resistance	[35]
13-wk old, high-fat diet	Adipose tissue inflammation Adiposity Fasting hyperleptinemia Fasting hyperlipidemia Fasting hypoadiponectinemia Insulin resistance	[36]

by Cbl-b-mediated ubiquitination and breakdown of toll/IL-1 receptor domain-containing adapter inducing interferon-β (TRIF) and MyD88, which are adaptor molecules for TLR4 signal transduction. This suppression by Cbl-b was dependent on the presence of integrin α_M (CD11b). In neutrophils, Cbl-b also suppresses LPS signaling by preventing the formation of the TLR4-MyD88 complex[53]. These reports suggest that Cbl-b is a critical regulator of the macrophage activation mediated by LPS-induced TLR4 signaling.

TLR4 activation by SFAs thought to play a pivotal role in ATM activation-induced IR. Diet-induced obesity increases the circulating levels of free FAs. SFAs directly induce IR in the liver, skeletal muscle and AT[54]. Furthermore, SFAs cause chronic inflammation through ATM activation, which is mediated by TLR4 signal transduction[25,26]. Recently, we demonstrated that the knockout of Cbl-b promoted and IR through ATM accumulation in HFD-fed mice[36]. In addition to increased ATM accumulation, inflammatory cytokine secretion was increased in the AT of obese Cbl-b$^{-/-}$ mice. In addition to aging, the consumption of a HFD increases MCP-1 expression in WAT. We found that depletion of Cbl-b in murine peritoneal macrophages promotes SFA-induced TLR4 signal transduction (Figure 3). Palmitate-induced JNK phosphorylation and IL-6 expression were enhanced in Cbl-b-deficient peritoneal macrophages. We also showed that TLR4 is a substrate for Cbl-b in the presence of SFAs. Overexpression of Cbl-b increased the ubiquitination and breakdown of TLR4 after palmitate treatment. Consistent with this finding, the TLR4 protein expression levels on the surface of Cbl-b-deficient peritoneal macrophages were increased. It is well known that LPS treatment induces the phosphorylation of 2 tyrosine residues of human TLR4[55]. The phosphorylation of TLR4 is required to activate signaling by promoting an interaction with Syk in macrophages[56]. It remains unknown whether SFAs also induce the TLR4 tyrosine phosphorylation in macrophages. Although LPS induces the ubiquitination and degradation of MyD88 and TRIF[8], SFAs do not induce these pathways in macrophages[36]. These differences between LPS and SFAs are not fully understood. Further investigations are needed to elucidate the mechanism of SFA-induced phosphorylation of TLR4.

Recently, Lu *et al*[57] reported that treatment with a TLR4 antagonist improves insulin sensitivity and macrophage accumulation in the atherosclerotic lesions of low-density lipoprotein receptor-deficient mice. We demonstrated the TLR4 signaling was strongly associated with the development of IR in obese Cbl-b$^{-/-}$ mice using eritoran, a TLR4 antagonist[58]. The eritoran treatment reduced the insulin sensitivity and glucose tolerance in obese Cbl-b$^{-/-}$ mice. This phenomenon may be caused by a decrease in ATM accumulation. In fact, we found that an anti-TLR4 antibody inhibited SFA-induced TLR4 signal transduction in murine peritoneal macrophages. Our data suggest that TLR4 antagonists are potent therapeutic drugs that can be used to treat the IR mediated by ATM activation.

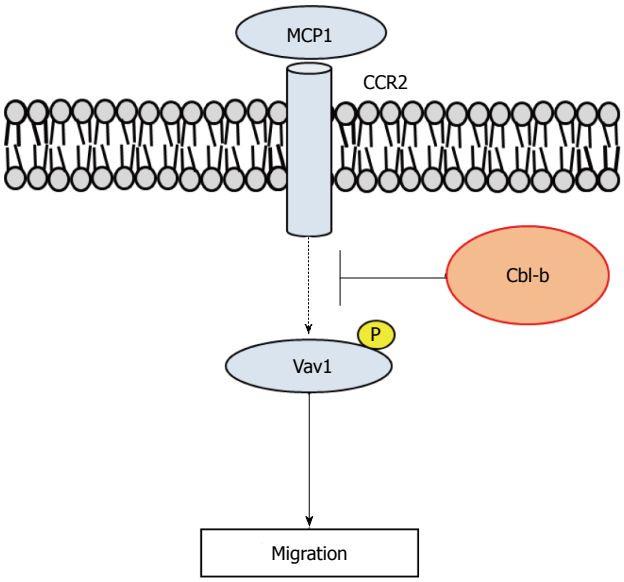

Figure 2 Casitas b-lineage lymphoma-b suppresses macrophage migration. Monocyte chemoattractant protein (MCP)-1 causes macrophages to infiltrate adipose tissue *via* C-C chemokine receptor 2 (CCR2). Phosphorylation (P) of Vav1 mediates macrophage migration, and Cbl-b negatively regulates macrophage migration by suppressing Vav1 phosphorylation.

CONCLUSION

Obesity causes various diseases through the development of IR, which is a clinical feature of patients with type 2 diabetes. Prediabetes is defined as impaired fasting glucose, impaired glucose tolerance and/or high levels of plasma glycated hemoglobin and is a critical risk factor for cardiovascular diseases[59]. AT inflammation is thought to be associated with the onset of prediabetes[60]. Therefore, to prevent type 2 diabetes, the development of ab effective therapeutic strategy for obesity-induced IR is urgently needed.

Aging- and diet-induced obesity causes the IR mediated by ATM activation. However, the mechanisms underlying ATM activation are poorly understood. We showed that Cbl-b reduces IR by suppressing macrophage migration and activation in mice. However, several questions remain about the biological implication of Cbl-b in human cells. The molecular mechanism underlying the effects of Cbl-b

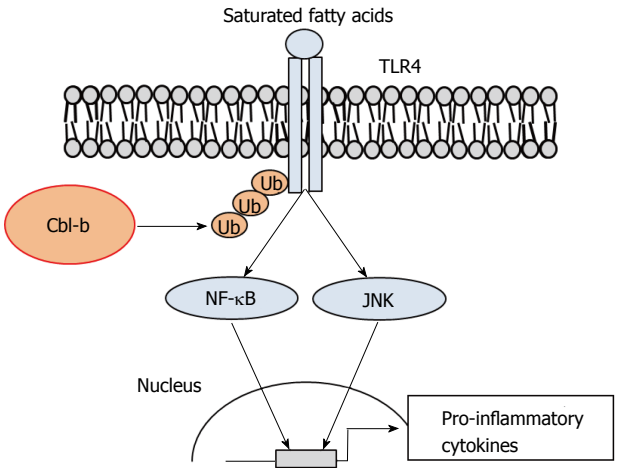

Figure 3 Casitas b-lineage lymphoma-b suppresses toll-like receptor 4 signaling in macrophages. Cbl-b negatively regulates saturated fatty acid (SFA)-induced TLR4 signal transduction. SFA-triggered TLR4 signaling induces the expression of inflammatory cytokines *via* JNK and NF-κB. The released inflammatory cytokines cause insulin resistance in the liver, skeletal muscle and adipose tissue. In the presence of SFAs, Cbl-b induces the ubiquitination and degradation of TLR4 in macrophages. Ub: Ubiquitin; TLR4: Toll-like receptor 4; Cbl-b: Casitas b-lineage lymphoma-b; JNK: Jun N-terminal kinase.

in macrophages is unknown. Further investigations are essential to identify new tyrosine kinases for Cbl-b. Recently, it was shown that macrophages infiltrate the fatty liver and AT in obesity. Cbl-b may suppress the macrophage activation in fatty liver. The side effects of Cbl-b activation remain unclear. We also showed that Cbl-b disturbed insulin-like growth factor signaling through ubiquitination and degradation of insulin receptor substrate-1 in skeletal muscle under unloading conditions[61]. Although we did not observe an enhancement of insulin signal transduction in lean Cbl-b$^{-/-}$ mice, tissue-specific Cbl-b activation may be important when using a drug delivery system, such as liposomes. A better understanding of Cbl-b-mediated ATM activation may provide the basis for developing novel therapeutic strategies that can be used to treat IR.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Statin, testosterone and phosphodiesterase 5-inhibitor treatments and age related mortality in diabetes

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Author contributions: The original study was designed by Hackett G; Strange RC and Ramachandran S have had an interest in the work of Gompertz B and conceived the idea of investigating age related mortality patterns in different cohorts and studying how interventions alter age related mortality; all authors contributed to the data analysis this work being supervised by Jones PW as well as drafting, revising and approving the final version of the manuscript.

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Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at (sud.ramachandran@heartofengland.nhs.uk). Participants gave informed consent for anonymous data to be shared with health authorities and published in scientific journals.

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Abstract

AIM

To determine how statins, testosterone (T) replacement therapy (TRT) and phosphodiesterase 5-inhibitors (PDE5I) influence age related mortality in diabetic men.

METHODS

We studied 857 diabetic men screened for the BLAST study, stratifying them (mean follow-up = 3.8 years) into: (1) Normal T levels/untreated (total T > 12 nmol/L and free T > 0.25 nmol/L), Low T/untreated and Low T/treated; (2) PDE5I/untreated and PDE5I/treated; and (3) statin/untreated and statin/treated groups. The relationship between age and mortality, alone and with T/TRT, statin and PDE5I treatment was studied using logistic regression. Mortality probability and 95%CI were calculated from the above models for each individual.

RESULTS

Age was associated with mortality (logistic regression, OR = 1.10, 95%CI: 1.08-1.13, $P < 0.001$). With all factors included, age (OR = 1.08, 95%CI: 1.06-1.11, $P < 0.001$), Low T/treated (OR = 0.38, 95%CI: 0.15-0.92, $P = 0.033$), PDE5I/treated (OR = 0.17, 95%CI: 0.053-0.56, $P = 0.004$) and statin/treated (OR = 0.59, 95%CI: 0.36-0.97, $P = 0.038$) were associated with lower mortality. Age related mortality was as described by Gompertz, $r^2 = 0.881$ when Ln (mortality) was plotted against age. The probability of mortality and 95%CI (from logistic regression) of individuals, treated/untreated with the drugs, alone and in combination was plotted against age. Overlap of 95%CI lines was evident with statins and TRT. No overlap was evident with PDE5I alone and with statins and TRT, this suggesting a change in the relationship between age and mortality.

CONCLUSION

We show that statins, PDE5I and TRT reduce mortality in diabetes. PDE5I, alone and with the other treatments significantly alter age related mortality in diabetic men.

Key words: Type 2 diabetes; Mortality; Gompertz-Makeham equation; Phosphodiesterase 5 inhibitors; Male hypogonadism; Statins; Testosterone replacement therapy

Core tip: We have described a study of men with type 2 diabetes showing that mortality rates are in accordance with the pattern described nearly 200 years ago by Benjamin Gompertz. The data show that statin, phosphodiesterase 5 inhibitors (PDE5I) and testosterone replacement in hypogonadal men reduce all-cause mortality. PDE5I, alone and in combination with the other 2 agents alters the association between age and mortality, thus improving prognosis. The graphical illustrations adopted in this paper communicate the impact of medical intervention very effectively to patients and this could potentially improve compliance leading to significant clinical benefit.

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INTRODUCTION

In 1825, Benjamin Gompertz described a law defining human mortality based on the equation, $\mu(x) = \alpha \times e^{\beta x}$, with $\mu(x)$ being the mortality rate at age x years and, α and β (considered to be the ageing rate) being constants[1]. Subsequently, Makeham proposed a modified Gompertz equation, $\mu(x) = \alpha \times e^{\beta x} + y$, that included a factor (y) describing extrinsic mortality thereby allowing the effect of age -independent and -dependent factors on mortality to be studied[1,2]. Avoidance of external factors such as conflict or starvation results in the age-independent component having less impact on death rate and therefore, mortality increases exponentially with age. It has been suggested that β is similar for all populations and it is the intercept (α) that varies[3]. In developed countries, the age-independent intercept is influenced by medical care as many treatments have a significant impact on mortality though factors encountered in early life can also influence later outcomes[4].

Mortality in type 2 diabetes (T2DM) is associated with age and therapy. Patients have a 1.5-2.5 fold higher mortality than the general population[5,6] with men and women suffering a mean 7.5 years and 7 years reduction in life expectancy respectively[7]. This increase in mortality appears related to the age at diagnosis, being lower in patients diagnosed their late 70 s compared with those aged in their mid-40 s[8].

Several inter-related pathologies are associated with mortality in T2DM and accordingly, statins and in men, testosterone replacement therapy (TRT) and phosphodiesterase 5 inhibitors (PDE5I) are commonly prescribed. Since rates of cardiovascular disease (CVD) associated mortality are 2-4 times higher than in non-diabetics, reduction of serum LDL-cholesterol is a key aim[9,10]. Statins are the mainstay of lipid lowering therapy with trials demonstrating marked reductions in CVD in T2DM patients[9,11]. Male hypogonadism characterised by low serum testosterone and sexual dysfunction is common in diabetics and also linked with mortality[12-15]. Studies in men with diabetes show low serum free testosterone is associated with carotid atherosclerosis identified by carotid artery intimal thickness and plaque score[16]. All-cause mortality is greater in those with low compared with normal total testosterone levels[17]. Three studies, two in men with T2DM, suggest TRT reduces all-cause mortality[17-19]. A randomized controlled study of men with T2DM showed TRT improves symptoms

of hypogonadism, the different symptoms significantly improving at varying testosterone thresholds[20]. Importantly, in T2DM patients, erectile dysfunction (ED) appears to be an independent predictor of vascular disease and mortality[13-15]. PDE5I have beneficial effects on endothelial function, insulin resistance and possess potentially cardio-protective properties[15,21,22]. Gazzaruso *et al*[15] showed that PDE5I use in T2DM men was associated with reductions in major adverse cardiac events and angiography confirmed coronary artery disease. Our longitudinal prospective study indicated that PDE5I use was possibly associated with reduced mortality independent of TRT and statin therapy[19]. Further, Anderson *et al*[23] reported a 31% reduction in all-cause mortality and 26% reduction in myocardial infarction in men with T2DM prescribed PDE5I.

We recently reported in a prospective longitudinal study of 857 men with T2DM that TRT and PDE5I were independently associated with increased survival in men with T2DM[19]. Importantly baseline body weight, body mass index, lipids, glycaemic control and hypertension were not associated with mortality. We now describe a retrospective analysis of this cohort determining the association between age and mortality, establishing whether the mortality rate follows the pattern described by Gompertz and estimating how testosterone status and treatments (statins, TRT and PDE5I) alter this relationship.

MATERIALS AND METHODS

This study is a retrospective analysis of follow-up data obtained in 857 men with T2DM screened following informed consent for the randomised double blind placebo controlled BLAST (Birmingham, Lichfield, Atherstone, Sutton and Tamworth) study designed to investigate the effects of long acting testosterone on clinical symptoms and metabolic parameters over a 30 wk treatment period[24]. The patients were listed in the registers of 5 English Midlands practices and initially screened for total testosterone (TT) and free testosterone (FT) during April 2007-April 2009. Based on total and free testosterone levels, the 857 men were classified as Normal T (total testosterone > 12 nmol/L and free testosterone > 0.25 nmol/L) or Low T (total testosterone $\leqslant$ 12 nmol/L or free testosterone $\leqslant$ 0.25 nmol/L) with a second measurement taken at least 2 wk later in men with TT $\leqslant$ 12 nmol/L, according to the Endocrine Society clinical practice guidelines[25]. The BLAST intervention study were approved by the West Midlands Regional Ethics Committee (reference: 08/H1208/30), the National Institute for Health Research (Birmingham and the Black Country Comprehensive Local Research Park - RM&G reference: 1268) and Warwickshire Primary Care Trust (reference: WAR230909) with the long term follow-up approved as an audit by all the appropriate Primary Care Trust Ethics Committees.

United Kingdom Primary care diabetes care treatment of glycaemic control, dyslipidaemia and hypertension is protocol and guideline driven as part of the Quality Outcomes Framework initiative. TRT was prescribed according to the BLAST study programme[24,26]. The United Kingdom NHS regulations allowed PDE5I prescribing for ED in men with diabetes with a suggested regime of 1 dose/wk[27].

The 857 men were categorised by statin, TRT and PDE5I treatment at death or final visit, firstly, by statin treatment; 195 men were Statin/untreated and 662 men Statin/treated, secondly by hypogonadism and TRT; 320 men were Normal T (TT > 12 nmol/L and FT > 0.25 nmol/L)/untreated, 362 men were Low T (TT $\leqslant$ 12 nmol/L or FT $\leqslant$ 0.25 nmol/L)/untreated and 175 men were Low T/treated (TT $\leqslant$ 12 nmol/L or FT $\leqslant$ 0.25 nmol/L)/treated and thirdly, by PDE5I treatment; 682 men were PDE5I/untreated and 175 men were PDE5I/treated. Mortality data was collected from the general practice databases, hospital letters and death certificates.

Laboratory methods

Statin and TRT prescribing was based on protocols based on laboratory measurements. Serum sex hormone binding globulin, albumin and lipids were analysed using a Roche Modular automated analyzer (Roche Diagnostics, Burgess Hill, United Kingdom). Early morning fasting TT was measured using the validated Roche common platform immunoassay. FT was calculated using the Vermeulen *et al*[28].

Stata version 8 (College Station, TX) was used for statistical analyses with all-cause mortality as primary end point. Differences in mortality between the alive and deceased groups were identified using χ^2 (statin, PDE5I, TRT and hypogonadism) and unpaired *t*-test (age at death or final visit). Logistic (and logit) regression was initially carried out to study the association between death/survival (dichotomous outcome) and age at death or final follow-up visit as the independent variable. Subsequently, separate models were developed with each treatment (statins, testosterone (status and treatment) and PDE5I) being included with age at death or final visit as the independent variables. Discrete ordinal variables were factorised with one category selected as reference. In the testosterone groups, Low T/untreated was selected as reference as Normal T/untreated and Low T/treated differed by one characteristic, *i.e.,* TT and FT concentration and TRT respectively. Patients on statins and PDE5I were compared to those not on treatment (reference groups). We estimated individual probability of mortality (and 95%CI for each man using separate logistic regression models. The statistical approaches used were reviewed by author, Professor Peter W Jones, Professor of Medical Statistics, Keele University, and considered appropriate.

RESULTS

Table 1 presents the data used to compare the age and treatment details of men who either survived or died during the study. The Table shows the mean age of the total study group and of the 754 men alive at study end

Table 1 Mortality in men with type 2 diabetes stratified by treatment with statins, testosterone status/treatment, phosphodiesterase 5-inhibitors and combinations of treatments *n* (%)

	Total group	Alive	Deceased	*P* value
Patient *n*	857	754	103	
Mean age at death or last visit/SD (yr)	67.4/11.6	66.2/11.3	76.2/10.2	< 0.0001[1]
Patient numbers (%) stratified by treatment				
Statin/untreated	195 (22.8)	162 (21.5)	33 (32.0)	0.017[2]
Statin/treated	662 (77.3)	592 (78.5)	70 (68.0)	
Normal T/untreated	320 (37.3)	284 (37.7)	36 (35.0)	< 0.001[2]
Low T/untreated	362 (42.2)	301 (39.9)	61 (59.2)	
Low T/treated	175 (20.4)	169 (22.4)	6 (5.8)	
PDE5I/untreated	682 (79.6)	582 (77.2)	100 (97.1)	< 0.001[2]
PDE5I/treated	175 (20.4)	172 (22.8)	3 (2.9)	
Not on any of the above therapeutic agents	125 (14.6)	92 (12.2)	33 (32.0)	0.002[2]
On all 3 therapeutic agents	45 (5.3)	43 (5.7)	2 (1.9)	

[1]Unpaired *t* test; [2]χ^2 analysis.

Table 2 Association between age and mortality corrected for statin treatment, testosterone status/treatment and phosphodiesterase 5-inhibitors treatment

	OR (95%CI)	*P* value
Model a		
Age (yr)	1.10 (1.08-1.13)	< 0.001
Model b		
Age (yr)	1.10 (1.07-1.13)	< 0.001
Statin/untreated	Reference	
Statin/treated	0.63 (0.39-1.01)	0.057
Model c		
Age (yr)	1.10 (1.07-1.12)	< 0.001
Normal T/untreated	0.61 (0.42-1.07)	0.092
Low T/untreated	Reference	
Low T/treated	0.31 (0.13-0.75)	0.009
Model d		
Age (yr)	1.09 (1.07-1.12)	< 0.001
PDE5I/untreated	Reference	
PDE5I/treated	0.16 (0.051-0.54)	0.003
Model e		
Age (yr)	1.08 (1.06-1.11)	< 0.001
Statin/untreated	Reference	
Statin/treated	0.59 (0.36-0.97)	0.038
Normal T/untreated	0.69 (0.43-1.10)	0.120
Low T/untreated	Reference	
Low T/treated	0.38 (0.15-0.92)	0.033
PDE5I/untreated	Reference	
PDE5I/treated	0.17 (0.053-0.56)	0.004

PDE5I: Phosphodiesterase 5-inhibitors.

and 103 deceased men. Mean age in the deceased group was significantly higher ($P < 0.0001$) than in survivors. Table 1 also shows the proportion of alive/deceased men treated with statin or PDE5I. In the deceased group, a significantly lower proportion of men were treated with statins (68.0%, $P = 0.017$) or PDE5I (2.9%, $P < 0.001$) compared with survivors (78.5%, 22.8% respectively). To assess the impact of hypogonadism and TRT on mortality, we stratified the 857 men into three groups; Normal T/untreated (eugonadal), Low T/untreated and Low T/treated. Table 1 shows in the deceased group that the proportions of men given TRT (5.8%, $P < 0.001$) or who were eugonadal (35.0%, $P = 0.037$) was significantly lower than that of men in the Low T/untreated group (59.2%).

Importantly, in two of the treatment groups the age at final visit of survivors varied; PDE5I treatment (PDE5I/untreated: Mean age = 67.2 ± 10.1 years, PDE5I/treated: Mean age = 62.7 ± 10.0 years, $P < 0.0001$) and TRT (Low T/untreated: 67.3 ± 11.3 years, Low T/treated: 61.8 ± 10.9 years, $P < 0.0001$) patients. No corresponding difference in age at final visit in survivors was observed in the Statin/untreated *vs* Statin/treated and Normal T/untreated *vs* Low T/untreated groups. Age at death did not significantly differ with statin (Statin/untreated: Mean age = 77.0 ± 10.5 years, Statin/treated: Mean age = 75.8 ± 10.1 years, $P = 0.56$) or PDE5I treatment (PDE5I/untreated: Mean age = 76.4 ± 10.1 years, PDE5I/treated: Mean age = 67.0 ± 13.3 years, $P = 0.11$). Importantly, only 3 patients on PDE5I treatment died during follow-up (Table 1). Interestingly, age at death varied between the testosterone groups (Normal T/untreated: Mean age = 73.9 ± 10.6 years *vs* Low T/untreated: Mean age = 78.4 ± 8.9 years, $P = 0.0.028$, Low T/untreated: Mean age = 78.4 ± 8.9 *vs* Low T/treated: Mean age 66.3 ± 13.1 years, $P = 0.0034$).

As age at death or final visit differed between the treatment and testosterone status groups we used logistic regression analyses to see if the associations in Table 1 were independent. Table 2 shows age is associated with mortality regardless of the other factors added to regression models (Models a-e). Significant reduction in mortality was observed with TRT (Low T men - Model c) and PDE5I (Model d) treatments while the benefit due to statins approached significance (Model b). All 3 treatments were significantly associated with decreased mortality when entered together (Model e).

We determined if our mortality data adhered to the Gompertz-Makeham equation. Figure 1 shows mortality rate (as % and logarithmic values) plotted against age (as 5 year categories). A linear relationship was observed between Ln (mortality) and age ($R^2 = 0.881$) suggesting that the mortality observed in our cohort did fit the pattern initially described by Gompertz.

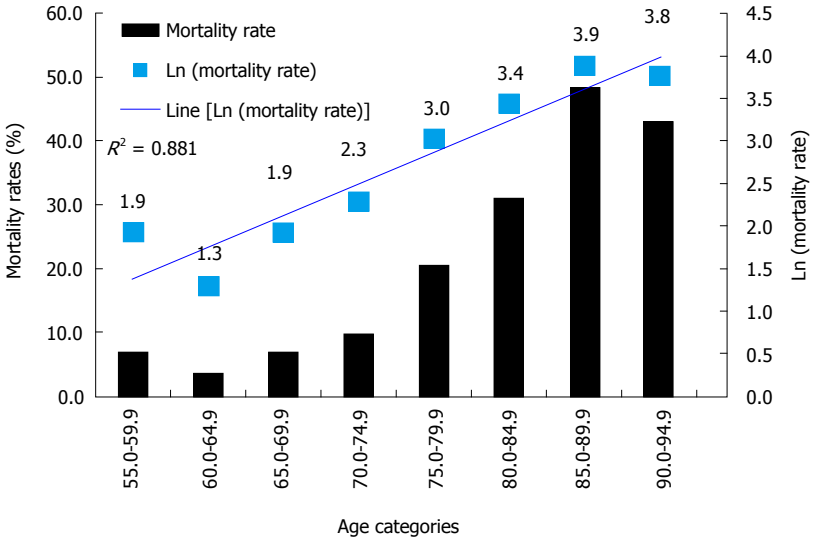
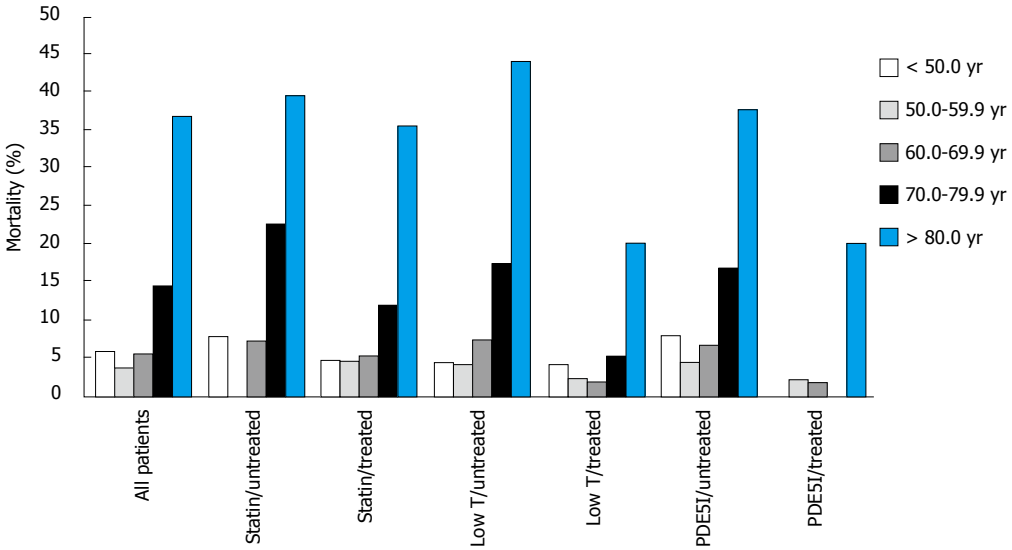


Figure 1 Association between Ln (mortality rate) and age.



Age (yr)	All patients	Mortality %	Statin/ untreated	Mortality %	Statin/ treated	Mortality %	Low T/ untreated	Mortality %	Low T/treated	Mortality %	PDE5I/ untreated	Mortality %	PDE5I/ treated	Mortality %
< 50.0	70	5.7	26	7.7	44	4.6	24	4.2	25	4.0	51	7.8	19	0.0
50.0-59.9	143	3.5	28	0.0	115	4.4	50	4.0	48	2.1	94	4.3	49	2.0
60.0-69.9	260	5.4	42	7.1	218	5.1	96	7.3	59	1.7	198	6.6	62	1.6
70.0-79.9	275	14.6	66	22.7	209	12.0	126	17.5	38	5.3	235	17.0	40	0.0
> 80.0	109	36.7	33	39.4	76	35.5	66	43.9	5	20.0	104	37.5	5	20.0

Figure 2 Mortality rates by age at death or final visit observed in the total group and treatment categories. PDE5I: Phosphodiesterase 5-inhibitors.

However, our cohort is heterogeneous as it comprises men on varying treatments that influence mortality (Table 1). Figure 2 displays the relationship between mortality rates in the total group and in the different treatment and age categories (5 years selected due to lower numbers in the subgroups). As predicted in the logistic regression analyses (Table 2), statin, TRT and PDE5I treatments reduced the age related mortality, though the reduction differed in the age categories.

To further graphically demonstrate the impact of statin, TRT and PDE5I on mortality, the probability of mortality of each patient together with the 95%CI were estimated from the logistic regression analyses in Table 2. Figures 3 show the probability of mortality plotted against the age of the patient in the total cohort, by the treatment groups and by treatment combination (men on all 3 treatments *vs* not on any of the treatments). In the statin (Figure 3B) and TRT (Figure 3C) plots some overlap in the 95%CI is seen between treated compared to untreated men. For PDE5I (Figure 3D) and combination treatments (Figure 3E) no overlap of 95%CI values was observed after 50 years of age indicating the relationship between mortality and age is significantly altered.

DISCUSSION

In a recent longitudinal study we showed that in men

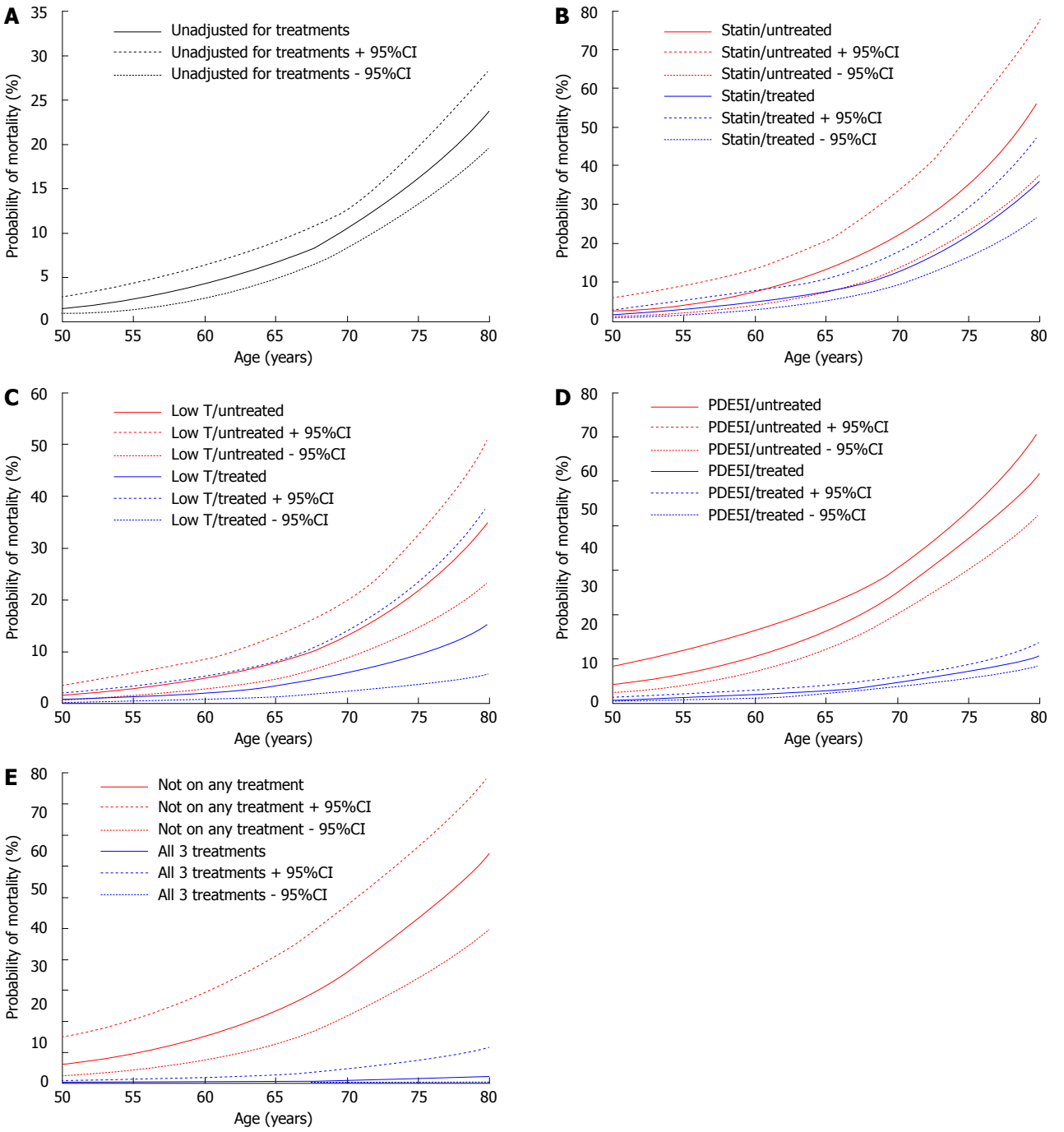


Figure 3 Association between probability of mortality and age. The estimated mortality probability and 95%CI from the fitted logistic regression (Table 2) were calculated from the logistic regression analyses seen in Table 2 and plotted against age at death or final visit in the following groups. Age was restricted to between 50-80 years due to reduced patient numbers in the treatment (Low T/treated and PDE5I/treated) groups (> 80 years) and the exponential pattern only being evident in the total group over the age of 50 years (Figure 1). A: Total group (from Model a in Table 2); B: Men stratified by statin treatment (from Model b in Table 2); C: Men stratified by testosterone treatment (from Model c in Table 2); D: Men stratified by PDE5I treatment (from Model d in Table 2); E: Men on all and none of the above treatments (from Model e in Table 2). PDE5I: Phosphodiesterase 5-inhibitors.

with T2DM, hypogonadism is associated with increased mortality compared to eugonadal men. Importantly TRT abolished this increase in mortality[19]. PDE5I (HR = 0.21, P = 0.009) and possibly statin (HR = 0.69, P = 0.086) use were also observed to reduce mortality[19]. Our aim in this paper was to determine how these three commonly used treatments influence the association between age and mortality in T2DM men. Our approach was to determine the probability of a patient in each treatment group living or dying at a particular age. Importantly, the Gompertz-Makeham law accurately describes the association between age and mortality in subjects aged approximately between 30-80 years, an age range that encompasses most of our study group.

Data on the United Kingdom Government Web Archive (http://webarchive.nationalarchives.gov.uk/20160

105160709/http://www.ons.gov.uk/ons/rel/vsob1/death-reg-sum-tables/2013/sty-mortality-rates-by-age.html - accessed 2016 Oct 24) shows firstly, the relevance of the Gompertz model; mortality rates between 1963 and 2013 demonstrate an exponential pattern similar to that described nearly 200 years ago by Gompertz. Secondly, the Archive data show that while the adult mortality rate had fallen during 1963-2013, possibly because of a reduction in CVD resulting from statin use and smoking cessation, it still demonstrated an exponential pattern in both genders.

In this study, we used a retrospective approach based on logistic regression to study the impact of treatment on the association between age and mortality. Accordingly, we compared results from this study with those from a previous prospective longitudinal analysis[19]. As expected, similar independent associations between the treatments and mortality were observed (Table 2, model e) enabling us to examine the impact of treatment on the relationship between age and probability of mortality. The relationship between age and mortality remained significant regardless of which (single or combination) treatment factors were added to regression models.

Life tables derived from data from an adult population will reflect a combination of phenotypes related to lifestyle, pathology, therapy and genetic factors that confer varying risks of dying at a particular age. Thus, we did not expect such a close fit (R^2 = 0.881) as that observed when the mortality rates of our total cohort were transformed logarithmically and plotted against age. It was tempting to carry out a similar exercise in the treatment and non-treatment subgroups but small patient numbers and low mortality rates in the testosterone and PDE5I treated men prevented this. Greater patient numbers with higher event rates would have permitted a study of these subgroups.

Using logistic regression to estimate probability of mortality for each individual patient allowed graphic demonstration of the impact of treatment on age, the most significant predictor of death. Whilst statin use and TRT did not show a statistically significant effect on the relationship between age and mortality, PDE5I treatment and combination (statin, TRT and PDE5I) treatment clearly did with no overlap of 95%CI. These results are compatible with studies showing that in T2DM patients, treatment with vardenafil results in improved endothelial parameters including flow-mediated dilation, interleukin-6 and testosterone levels[29] and indicate that a randomised controlled trial (RCT) is required for PDE5I and TRT in men with T2DM. It would be interesting if some of the large RCTs carried out showing statin-associated reductions in all-cause mortality such as 4S were analysed to establish ways in which the relationship between age and mortality may have been altered.

There are limitations to our study. The TRT arm was based on an intention to treat. The age of onset and duration of diabetes as well as exposure to statins and PDE5I were not documented. Data on the type of drug and dose was not completely recorded. We assumed that statin and PDE5I treatments were protocol driven. However, it is possible that patient selection, especially with PDE5I prescribing existed. It is believed however, that the principal reason for PDE5I prescribing is ED which has been established as a significant predictor of CVD and all-cause mortality.

Despite their limitations, our findings are important. We showed that mortality rates in men with T2DM follow the pattern described by Gompertz. We confirmed that statin, TRT and PDE5I reduce mortality in this cohort and have described how they influenced the relationship between age and mortality. We believe that our approach of communicating the effectiveness of an intervention by determining the probability of mortality at different ages is easy to understand and could be used by clinicians to improve patient compliance and lead to clinical benefit.

Our study examines the relationship between age and mortality in men with diabetes. Age was related to mortality in accordance with the Gompertz-Makeham law. We show that statin, TRT and PDE5I treatments impacted all-cause mortality and PDE5I treatment alone and in combination with statin and TRT significantly altered the relationship between age and mortality.

COMMENTS

Background

As far as the authors are aware the Gompertz-Makeham association has not been used to demonstrate the impact of medical treatments on the relationship between age and mortality.

Research frontiers

There is considerable debate regarding the effects of testosterone replacement therapy and phosphodiesterase 5-inhibitors (PDE5I) treatment in diabetic men. Although no randomised controlled studies exist longitudinal observational studies have shown potential benefits.

Innovations and breakthroughs

Having established that statin, PDE5I treatment and testosterone replacement therapy reduced mortality in the cohort, the studied the influence of these agents on this association. The data are presented in a novel graphical manner, clearly demonstrating the impact of these agents on mortality. The authors suggest the graphical illustrations in this paper will communicate the benefit of these interventions to patients and have a major positive bearing on patient compliance.

Peer-review

This paper is a very interesting retrospective study investigating whether the mortality rate follows the pattern described by Gompertz and estimating how testosterone status and treatments (statins, testosterone replacement therapy and phosphodiesterase 5 inhibitors) alter the mortality rate. A substantial and extremely meticulous work has been done and the findings are consistent.

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ORIGINAL ARTICLE

Prospective Study

Role of angiotensin converting enzyme and angiotensinogen gene polymorphisms in angiotensin converting enzyme inhibitor-mediated antiproteinuric action in type 2 diabetic nephropathy patients

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Author contributions: Kalra OP, Madhu SV, Banerjee BD and Tripathi AK were involved in planning and designing the research work; Kalra OP, Madhu SV, Yadav A and Raizada A contributed to the enrollment and medication of patients; Aggarwal N, Kare PK and Varshney P were involved in the biochemical investigations and data collection; Aggarwal N carried out data interpretation and drafted the manuscript; Tripathi AK, the corresponding author, was involved in overall supervision and revision of the manuscript.

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Abstract

AIM

To investigate the role of genetic variants of angiotensin converting enzyme (ACE) and angiotensinogen (AGT) genes in the antiproteinuric efficacy of ACE inhibitor therapy in diabetic nephropathy (DN) patients.

METHODS

In the present study, 270 type 2 diabetes mellitus patients with nephropathy were enrolled and treated with ACE inhibitor (ramipril) and followed at 6 mo for renal function and albumin excretion by estimating serum creatinine, end stage renal disease, and albumin/creatinine ratio (ACR) in urine. Genotyping of ACE I/D and AGT M235T polymorphisms were performed by using primer specific polymerase chain reaction (PCR) and PCR-RFLP techniques, respectively.

RESULTS

Forty-eight percent of DN patients (responders) benefited with respect to proteinuria from ACE inhibitor therapy at 6 mo follow-up. A significant reduction in ACR was observed after 6 mo treatment with ACE inhibitor irrespective of whether DN patients were micro-albuminuric (≥ 30 and < 300 mg/g creatinine) or macro-albuminuric (≥ 300 mg/g creatinine) at the time of enrollment. However, macro-albuminuric patients (55%) showed better response to therapy. A reduction in urinary ACR was found independent of genotypes of ACE I/D and AGT M235T polymorphisms although macro-albuminuric patients having TT genotype showed statistically insignificant increased response (72%).

CONCLUSION

ACE inhibitor therapy reduced urinary ACR by ≥ 30% in 50% of DN patients and the response is independent of ACE I/D and AGT M235T polymorphisms.

Key words: Diabetic nephropathy; Angiotensin converting enzyme inhibitor therapy; Renin-angiotensin-aldosterone system gene polymorphisms; Responder; Urinary albumin/creatinine ratio; Albuminuria

Core tip: Angiotensin converting enzyme (ACE) inhibitors are used as standard therapy in patients with diabetic nephropathy (DN) and reported to have reno-protective effect in these patients; however, the response to ACE inhibitor therapy is not uniform in all patients. We investigated whether ACE I/D and angiotensinogen gene (AGT) M235T polymorphisms of genes of the renin-angiotensin-aldosterone system are associated with variable response to ACE inhibitors in DN patients. ACE inhibitor treatment in DN patients caused a significant reduction in urinary protein excretion and was found independent of ACE I/D and AGT M235T polymorphisms.

Aggarwal N, Kare PK, Varshney P, Kalra OP, Madhu SV, Banerjee BD, Yadav A, Raizada A, Tripathi AK. Role of angiotensin converting enzyme and angiotensinogen gene polymorphisms in angiotensin converting enzyme inhibitor-mediated antiproteinuric action in type 2 diabetic nephropathy patients. *World J Diabetes* 2017; 8(3): 112-119 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i3/112.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i3.112

INTRODUCTION

Diabetic nephropathy (DN) is a clinical syndrome that occurs approximately in 20%-30% of patients with diabetes mellitus (DM). Nephropathy gradually progresses and makes the patient dependent on renal replacement therapy. DN patients clinically present with persistent micro-albuminuria (≥ 30 to 299 mg/g creatinine) which subsequently progresses to macro-albuminuria (≥ 300 mg/g creatinine)[1]. Later severity of the disease is characterized by a fall in estimated glomerular filtration rate (eGFR) as a consequence of renal impairment, ultimately leading to end stage renal disease (ESRD)[2]. Various factors including poor glycemic control, family history of diabetes or hypertension may predispose to the development of DN; however; not all DM patients tend to develop nephropathy[3].

The renin-angiotensin-aldosterone system (RAAS), which plays an important role in regulating blood pressure, is involved in the pathophysiology of renal complications including DN. Polymorphisms of various genes of RAAS, particularly angiotensin converting enzyme (ACE) and angiotensinogen (AGT) genes, have been strongly implicated in the development and progression of nephropathy[4,5]. ACE is a zinc-dependent di-peptidase enzyme which catalyzes the conversion of inactive angiotensin (angiotensin-Ⅰ) to angiotensin-Ⅱ[6]. The *ACE* gene is located at the 17q23 locus and known to be associated with the pathogenesis of DN, including progression to overt proteinuria. The *ACE* gene is highly polymorphic in nature. Of the 160 polymorphisms known, insertion/deletion (*I/D*) polymorphism is the most studied as it affects ACE enzyme activity in blood. *I/D* polymorphism involves the presence or absence of a 287 bp Alu repeat in intron 16 of the gene. It has been observed that DD genotype is associated with higher ACE activity and II genotype is associated with the lowest ACE activity[7].

The *AGT* gene (*rs 699*) is located at chromosome 1 and consists of five exons, and it has more than 23 variants[8]. The common polymorphism of the *AGT* gene is *M235T*, which encodes threonine instead of methionine at position 235 in exon 2[9]. T allele of the *M235T* variant is associated with a higher plasma AGT level[10].

A number of drugs that block the RAAS like ACE inhibitors and angiotensin receptor blockers (ARB) are often prescribed to control hypertension; in addition, these drugs are known to control proteinuria either alone or in combination in DN patients[11]. However, the reno-protective response to ACE inhibitor therapy is not uniform in all patients. The reasons behind the uneven antiproteinuric response to these drugs are not completely understood. The polymorphisms of genes of RAAS may be possibly involved in this process.

Despite several studies on association of *ACE* and *AGT* gene polymorphisms with ACE inhibitor treatment in type 2 DM (T2DM) patients with nephropathy, no substantial data are available on the role of *ACE* and *AGT* gene polymorphisms in antiproteinuric efficacy of ACE

inhibitors in the Indian context. In the present study, we examined the association of *ACE* and *AGT* gene polymorphisms with antiproteinuric response to ACE inhibitor therapy in north Indian type 2 diabetic patients with nephropathy.

MATERIALS AND METHODS

Subjects

This study was designed as a single arm prospective longitudinal study to evaluate the antiproteinuric effect of ACE inhibitor therapy based on change in albumin/creatinine ratio (ACR), with the baseline data serving as reference values (control). The required number of cases for 80% power at 5% type Ⅰ error in detecting a reduction of proteinuria to at least 30% of pretreatment value for a given odds ratio of 1.5 is 221, based on the frequency of mutant *ACE* gene allele in the Asian population as 40%[12]. In order to accommodate drop out during the course of the study, we recruited 270 patients with T2DM having persistent microalbuminuria (30-300 mg/g creatinine) or overt albuminuria (> 300 mg/g creatinine), of whom 18 could not complete the follow-up. The patients were enrolled from Department of Medicine, Diabetic and Nephrology Clinic at Guru Teg Bahadur Hospital, Delhi, India. Patients having an age between 30 to 65 years and a duration of diabetes ⩾ 5 years, with the evidence of diabetic retinopathy and stages 1 to 3 chronic kidney disease (CKD), were recruited. Patients intolerant to ACE inhibitors, pregnant or lactating women, patients taking aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) were excluded from the study. Diagnosis of DM was based upon the 2012 American Diabetes Association (ADA) guidelines. Patients having evidence of 1+ or more proteinuria by urinary dipstick test were included in the study. In addition, patients with dipstick negative proteinuria were screened by urinary dipstick for the presence of microalbumin. Patients with evidence of micro-albuminuria or overt proteinuria on two separate occasions at least 6 wk apart were included in the study and assessed for urinary ACR.

The study was approved by Institutional Ethics Committee-Human Research (IEC-HR) of University College of Medical Sciences and written informed consent was obtained from all patients. All enrolled patients were under satisfactory glycemic control and were under well-controlled blood pressure. The patients were followed after 6 mo of initiation of ACE inhibitor therapy. All were treated initially with ramipril 5 mg/d along with anti-diabetic therapy. The dose was up-titrated to a maximum of 20 mg/d at one or two equally divided doses.

Clinical response assessment

The decrease in urinary ACR (ACR%) was calculated as (baseline value - follow-up value) × 100/baseline value.

Patients were classified as responders when they had a decrease in urinary ACR ⩾ 30% or as non-responders when they had a decrease in urinary ACR > 30% at the end of 6 mo follow-up[13,14].

Measurement of biochemical parameters

Blood samples (5 mL) were collected for biochemical analysis and genotype study. Blood was centrifuged at 1000 g for 15 min for serum separation. Serum samples were frozen at -80 ℃ until assayed. All parameters were determined within a month after sample collection. Morning spot urine samples were collected for urine albumin and urine creatinine tests.

The plasma glucose level was measured by glucose oxidase-peroxidase method and quantified spectrophotometrically at 500 nm. HbA1c was estimated by micro-column based technique and quantified spectrophotometrically at 500 nm. Total cholesterol (TC), serum sodium, potassium and hemoglobin were determined using routine clinical assays in hospital laboratory. Average of three blood pressure readings taken 15 min apart was calculated, and all patients underwent fundus examination for the detection of diabetic retinopathy.

Urine and serum creatinine levels were estimated by alkaline picrate Jaffe's method (kinetic method). Urine albumin was measured by an immuno-turbidometric assay (Nephelometer, Nephstar) after calibration of the instrument by the standard provided. The minimum sensitivity is 10 mg/L. The result is expressed as ACR in terms of mg/g creatinine.

Determination of genotypes

ACE I/D gene polymorphism: The *ACE* gene (*rs 4646994*) *I/D* polymorphism was determined by polymerase chain reaction (PCR) using a flanking primer pair that recognizes the insertion-specific sequence. The 25 μL PCR reaction mixture contained 100 ng of genomic DNA and amplification buffer containing 20 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of dNTPs, 10 pmol of each primer, and 1.0 U of Taq DNA polymerase (RBC, India). The DNA was amplified by cycling at 94 ℃ for 2 min, at 60 ℃ for 45 s, and at 72 ℃ for 2 min (Eppendorf PCR machine, Germany). After 30 cycles, the reaction was extended for an additional 8 min at 72 ℃. The oligonucleotide sequences of the primers were: 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGTGGCCATCACATTCGTCAGAT-3'. The PCR products were separated by 1.5% agarose gel electrophoresis, and a 490 bp band with insertion (I allele) and a 190 bp band with deletion (D allele) were visualized with ethidium bromide staining in the UVP Bio-Documentation System.

AGT M235T gene polymorphism: The *AGT* gene (*rs 699*) *M235T* polymorphism was determined by PCR-restriction fragment length polymorphism (PCR-RFLP) assay. The 25 μL PCR reaction mixture contained 100 ng of genomic DNA and amplification buffer containing 20 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L of dNTPs, 10 pmol of each primer, and 1.0 U of Taq DNA polymerase (RBC, India). The DNA was amplified by cycling at 94 ℃ for 1 min, at 68 ℃ for 45 s, and at 72 ℃

Table 1 Demographic and clinical characteristics of patients

Parameter	Type 2 diabetes mellitus with nephropathy
Number of patients (*n*)	270
Gender (male/female)	128/142
Age (yr)	52.23 ± 6.01[1]
Duration of diabetes (yr)	8.31 ± 3.09[1]
Family history of diabetes (yes/no)	105/165
Family history of hypertension (yes/no)	63/207
Medications	
Insulin (yes/no)	115/155
Metformin (yes/no)	153/117
Glimiperide (yes/no)	129/141

[1]Data are presented as mean ± SD.

for 2 min (Eppendorf PCR machine, Germany). After 30 cycles, the reaction was extended for an additional 10 min at 72 ℃. The oligonucleotide sequences of the primers were: 5′-CCGTTTGTGCAGGGCCTGGCTCTCT-3′ and 5′-CAGGGTGCTGTCCACACTGGACCCC-3′.

The PCR product was digested with restriction enzyme Tth111 I (Fermentas) to identify the M/T polymorphism at 37 ℃ for 16 h. Digested DNA fragment products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The presence of an uncut 165 bp band indicated homozygous MM genotype, 141 bp and 24 bp bands indicated TT homozygous genotype, and 165 bp, 141 bp and 24 bands indicated MT heterozygous genotype.

Statistical analysis

The statistical methods of this study were reviewed by Department of Biostatistics, UCMS and GTB Hospital, Delhi, India. Data of all the parameters were collected at enrollment and at 6 mo after ramipril treatment. Analyses of obtained data were performed by using SPSS, version 20.0. *P*-values < 0.05 were considered significant. χ^2 test was applied to compare genotype data of *ACE* and *AGT* genes in all groups. For biochemical parameters, paired student's *t*-test was applied to compare the baseline values with the values obtained at 6 mo. ACR values follows the skewed distribution, hence we applied non-parametric method (Wilcoxon-signed rank test) to compare the baseline ACR values with the values obtained at 6 mo.

RESULTS

Demographic and biochemical data at baseline and at 6 mo after ACE inhibitor therapy

The demographic and biochemical data are listed in Tables 1 and 2. The age of the patients ranged from 30 to 60 years. The duration of diabetes ranged from 5 years to 20 years and mean duration of diabetes was 8.31 years. Approximately 39% of enrolled patients had a family history of diabetes and 23% had a family history of hypertension. Biochemical data before treatment and after 6 mo of treatment with ramipril are listed in Table 2. There was no significant change in blood urea, serum sodium, serum potassium, fasting plasma glucose, post prandial plasma glucose, systolic and diastolic blood pressure, hemoglobin or HbA1c level after follow-up. Also, the differences in serum creatinine and eGFR levels after treatment were not statistically significant.

Antiproteinuric effect of ACE inhibitor therapy

The antiproteinuric effect of ACE inhibitor therapy was evaluated by urinary ACR values. Patients with a decrease of more than 30% in ACR values were considered as responders to ACE inhibitor treatment. ACR values of enrolled patients at baseline varied widely and ranged from 30 to 14573 mg/g creatinine. An overall significant decrease in ACR values was observed after ACE inhibitor treatment as compared to baseline values (Table 3). Taken together, 48% of enrolled patients were found as responders to ACE inhibitor therapy. Subsequently, based on the ACR, patients were grouped as micro-albuminuric (ACR $\geqslant$ 30 and $\leqslant$ 300 mg/g creatinine) and macro-albuminuric (ACR > 300 mg/g creatinine). A significant decrease in ACR was observed in both the micro- and macro-albuminuric DN groups. In the micro-albuminuric DN group (n = 170), the percentage of responders was 45% whereas in the macro-albuminuric group (n = 82), the percentage was 55% at 6 mo follow-up.

Distribution of genotypes of ACE and AGT genes

ACE *I/D* polymorphism was studied by sequence specific PCR method and AGT *M235T* polymorphism was studied by PCR-RFLP method. Genotype distribution and allele frequency for *ACE* and *AGT* genes are listed in Table 4. Distribution of all genotypes was in Hardy-Weinberg equilibrium for all the subgroups of *ACE* and *AGT* genes. For *ACE* gene, the genotype frequency of II, ID, and DD was found to be 31%, 53% and 16%, respectively. For *AGT* gene, the genotype frequency of MM, MT, and TT was found to be 25%, 53% and 22%, respectively.

ACE and AGT polymorphisms and response to ACE inhibitor therapy

Table 5 shows the genotype distribution of DN patients based on the response to ACE inhibitor therapy. No significant change in the genotype distribution was observed among responders and non-responders with regard to *ACE* and *AGT* genes. When the patients were grouped as micro- and macro-albuminuric based on their ACR values (Table 6), no inter-genotype differences were observed in subgroups. However, macro-albuminuric patients carrying ACE *I/D* genotypes were responding in a better way to therapy compared with micro-albuminuric patients. Seventy-two percent of macro-albuminuric patients having TT genotype responded to therapy, although the difference was not statistically significant.

DISCUSSION

In the present study, we examined the antiproteinuric

Table 2 Biochemical parameters before and after treatment with angiotensin converting enzyme inhibitor

Parameter	Baseline[1]	6 mo[1,2]	*P*-value
No. of patients	*n* = 252	*n* = 252	
Blood urea (mmol/L)	2.22 ± 0.86	2.01 ± 0.77	0.661
Serum creatinine (μmol/L)	95.47-30	99-28.28	0.068
Serum sodium (mmol/L)	139.47 ± 4.11	135.14 ± 3.88	0.512
Serum potassium (mmol/L)	4.32 ± 0.65	4.30 ± 0.52	0.141
eGFR (MDRD) mL/min per 1.73 m^2	73.65 ± 24.71	68.90 ± 24.44	0.081
eGFR (EPI) mL/min per 1.73 m^2	73.40 ± 22.8	70.56 ± 21.30	0.07
Fasting plasma glucose (mmol/L)	7.63 ± 0.60	6.693 ± 0.81	0.08
Post-prandial plasma glucose (mmol/L)	10.33 ± 1.62	8.52 ± 1.3	0.076
HbA1c (%)	6.52 ± 1.71	6.1 ± 1.14	0.06
Hemoglobin (g/L)	123.8 ± 23	111.2 ± 31	0.65
Systolic blood pressure (mmHg)	132.30 ± 13.67	130.12 ± 10.46	0.71
Diastolic blood pressure (mmHg)	86.10 ± 10.03	84.07 ± 8.32	0.68

[1]Data are presented as mean ± SD; [2]*P* > 0.05. HbA1c: Hemoglobin A1c; eGFR: Estimated glomerular filtration rate.

Table 3 Responders and non-responders before and after treatment with angiotensin converting enzyme inhibitor therapy

Patients	Urinary ACR at baseline[1]	Urinary ACR at 6 mo	*P*-value	R^2 (%)	NR (%)
Overall (*n* = 252)	185.97 (55.66-222.20)	118.64 (96.24-146.26)	< 0.001	121	131
Micro-albumin (*n* = 170)	78.79 (71.30-87.07)	53.67 (44.46-64.79)	< 0.001	76	94
Macro-albumin (*n* = 82)	1068.7 (879.62–1298.28)	596.45 (451.60-787.68)	< 0.001	45	37

[1]Median (IQR); [2]A decline of > 30% in ACR value at 6 mo is considered as R. R: Responders; NR: Non-responders; ACR: Albumin/creatinine ratio.

Table 4 Genotype distributions and allele frequency for angiotensin converting enzyme and angiotensinogen gene polymorphisms

Gene	*n* = 252	Genotype/allele	Percentage (%)
ACE (I/D)	Genotypic	II	31
	frequency	ID	53
		DD	16
	Allele	I	57
	frequency	D	43
AGT (M235T)	Genotypic	MM	25
	frequency	MT	53
		TT	22
	Allele	M	51
	frequency	T	49

ACE: Angiotensin converting enzyme; AGT: Angiotensinogen.

effect of ACE inhibitor (ramipril) in DN patients by following urinary ACR. ACE inhibitors are commonly used for inhibition of the RAAS and are known to have renoprotective efficacy in both diabetic and non-diabetic kidney diseases[15] and antiproteinuric efficacy of ACE inhibitors are more pronounced than any other antihypertensive drugs[16]. However, there are variable responses regarding antiproteinuric efficacy of RAAS blockers among patients and a 20%-80% reduction was observed[17]. In the present study, overall we observed a 36% reduction in ACR values and about 48% of patients responded to therapy. Our finding is in accordance with previous studies showing overall decrease in albumin excretion after treatment with ACE inhibitor[13,18-20]. According to the NKF KDOQI guidelines[14], ACE inhibitors reduced protein excretion by approximately 35% to 40%, which is greater than other antihypertensive agents when the effect of blood pressure has been taken into account. Hence, in the present study patients with an ACR change ⩾ 30% were considered as responders to ACE inhibitor therapy. When subdividing our study subjects as micro- and macro-albuminuric, it was observed that 55% of patients with macro-albuminuria responded in a better way to ACE inhibitor therapy. Earlier antiproteinuric effect of ACE inhibitor has been shown to be more pronounced in macro-albuminuric patients[21,22]. The mechanism leading to the antiproteinuric effect of ACE inhibitors has not been elucidated fully. However, it is thought that ACE inhibitors cause efferent arteriolar vasodilation of glomerulus and thereby decrease the intraglomerular hypertension, leading to anti-proteinuric effect[23]. Recently it has been shown that ACE inhibitors ameliorate the glomerular membrane size-selective dysfunction, thus resulting in anti-proteinuric effect[24].

In order to find out the reason behind differential responses to ACE inhibitor therapy in DN patients, we studied the polymorphisms of *ACE* and *AGT* genes as these polymorphisms are strongly associated with the progression of DN. The genotype distribution of *ACE* gene observed in our study subjects is in line with most of the previous studies on the Indian population[25,26].

In the present study, the percentage of responders did not differ significantly with regard to *ACE I/D* genotypes, indicating that the antiproteinuric effect of ACE inhibitors is independent of *ACE* genotype. Similarly, the finding that the anti-proteinuric effect of ACE inhibitors is independent of *ACE* genotypes has been reported by

Table 5 Genotypic distribution of responders and non-responders

Gene	Genotype	No. of patients (n = 252)	At 6 mo follow-up		P-value[1]
			R (%) (n = 121)	NR (%) (n = 131)	
ACE (I/D)	II	78	38 (49)	40 (51)	0.893
	ID	133	62 (47)	71 (53)	
	DD	41	21 (51)	20 (49)	
AGT (M235T)	MM	61	34 (56)	27 (44)	0.369
	MT	134	59 (44)	75 (56)	
	TT	57	28 (49)	29 (51)	

[1]P > 0.05: Comparison between R or NR. ACE: Angiotensin converting enzyme; AGT: Angiotensinogen; R: Responders; NR: Non-responders.

Table 6 Genotypic distribution of responders and non-responders having micro-/ macro-albuminuria

Gene	Genotype (n = 252)	Micro-albuminuric group (n = 170)			Macro-albuminuric group (n = 82)		
		R (%) (n = 76)	NR (%) (n = 94)	P-value[1]	Rc (%) (n = 45)	NR (%) (n = 37)	P-value[2]
ACE (I/D)	II	23 (45)	28 (55)	0.974	15 (56)	12 (44)	0.636
	ID	42 (44)	53 (56)		20 (53)	18 (47)	
	DD	11 (49)	13 (54)		10 (59)	7 (41)	
AGT (M235T)	MM	25 (60)	17 (40)	0.11	9 (47)	10 (53)	0.201
	MT	36 (40)	53 (60)		23 (51)	22 (49)	
	TT	15 (38)	24 (62)		13 (72)	5 (28)	

[1]P > 0.05: Comparison between responders and non-responders to therapy in micro-albuminuric group; [2]P > 0.05: Comparison between responders and non-responders to therapy in macro-albuminuric group. ACE: Angiotensin converting enzyme; AGT: Angiotensinogen; R: Responders; NR: Non-responders.

several authors[14,27,28]. So *et al*[29] have reported that *ACE* Ⅱ genotype with a cumulative genetic risk score of < 1 in normoalbuminuricT2DM patients, is coupled with better response to ACE inhibitors, although no significant difference was found in renoprotective effect of ACE inhibitor therapy based on *ACE I/D* genotypes after 3 years of follow-up. The antiproteinuric effect of RAAS inhibitors in patients with macro-albuminuria is also found to be independent of *ACE I/D* genotypes[30]. However, there are a number of controversies about the association of *ACE I/D* genotypes with the therapeutic efficacy of ACE inhibitors. In Korean and Caucasian patients, DD genotype has been shown to be more responsive to ACE inhibitor therapy[31,32]. However, Japanese, European and Caucasian DN patients carrying II allele exhibit better reno-protection to ACE inhibitor therapy[33-35].

Another important gene of the RAAS is *AGT*, and *M235T* polymorphism influences the risk of nephropathy in T2DM patients[36,37]. Frequencies of M/T genotypes of the *AGT* gene in our study are similar to those reported by several other studies in different populations[36,38-41]. We observed that the percentage of responders did not differ significantly in different genotypes of the *AGT* gene, as compared to non-responders. This indicates that the antiproteinuric effect of ACE inhibitors is independent of genotypes of the *AGT* gene. When patients were subdivided as micro- and macro-albuminuric, we observed that macro-albuminuric patients carrying TT genotype showed better antiproteinuric response to ACE inhibitor therapy, although the result was not statistically significant. No significant reports are available on *AGT M235T* gene polymorphism and antiproteinuric response to ACE inhibitor therapy. Similar to our finding, reports by several authors failed to show any significant association between *AGT* polymorphism and diabetic chronic kidney disease[40,41]. Also no association was reported between *AGT M235T* genotypes and reduction in albumin excretion after ACE inhibitor treatment[29]. However, Narita *et al*[41] concludes that the therapeutic efficacy of ACE inhibitors or ARBs is influenced by *AGT M235T* genotypes in patients with IgA nephropathy.

Our study has several limitations. Patients were given different doses of ramipril as per their requirement of dose titration. In addition, short duration of follow-up period as well as heterogeneity in gender may also have hindered the significant association of *ACE I/D* or *AGT M235T* genotypes.

In conclusion, ACE inhibitor treatment in DN patients appears to cause a significant reduction in urinary protein excretion and macro-albuminuric patients exhibit better response. The antiproteinuric effect of ACE inhibitor therapy in patients is independent of *ACE I/D* and *AGT M235T* genotypes. Long term follow-up of larger populations with ACE inhibitor therapy may validate the present findings.

COMMENTS

Background

Angiotensin converting enzyme (ACE) inhibitors are the standard therapy for patients with hypertension, proteinuria and kidney diseases. The use of ACE inhibitors delays the progression of diabetic and non-diabetic kidney diseases. Various polymorphisms of the renin-angiotensin-aldosterone system (RAAS) have been implicated in the pathology of diabetic nephropathy. Of these,

polymorphism of the *ACE* gene is the most important. The current study was designed to evaluate the therapeutic efficacy of ACE inhibitor in terms of proteinuria and the role of *ACE* and *AGT* gene polymorphisms in ACE inhibitor-mediated antiproteinuric response in diabetic nephropathy patients.

Research frontiers

Patients on ACE inhibitor therapy have improved proteinuria. In this study, the authors observed that *ACE* and *AGT* gene polymorphisms do not have any role in reducing albuminuria in patients with diabetic nephropathy.

Innovations and breakthroughs

The literature suggests a mixed role of *ACE* gene polymorphisms in reno-protective action in diabetic patients. However, the present study suggests no role of *ACE I/D* and *AGT M235T* gene polymorphisms in modulating the renoprotective efficacy of ACE inhibitors in terms of reducing albuminuria in diabetic nephropathy patients.

Applications

The authors' study provides additional evidence supporting the therapeutic role of ACE inhibitors in reducing albuminuria. They conclude that genotypes of various genes of RAAS are not responsible for non-uniform response to ACE inhibitors in DN patients.

Terminology

Diabetic nephropathy: It is the damage to kidneys due to diabetes; Polymorphism: The presence of genetic variation within a population.

Peer-review

This is a good paper.

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REVIEW

Type 2 diabetes and quality of life

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Abstract

It is true that a primary goal of diabetes early diagnosis and treatment is quality of life (QoL). The term QoL is still confusing but it is agreed that it composes of four components: The physical component, mental, cogitative component, psychological and social component. Many articles have been written addressing those four components. During the last five years 15500 articles and reviews have been written addressing diabetes and coronary arterial disease, 16100 addressing diabetes and renal function, 28900 addressing diabetes and retinopathy, 16800 addressing diabetic foot ulcers and other 26300 addressing diabetic neuropathy. Moreover 17200 articles are dealing with diabetic sexual dysfunction, 24500 with the correlation of diabetes and depression 17500 about diabetes and dementia, only 1 about diabetes and family functioning and 1950000 about diabetes and QoL, indicating the worldwide interest. In order to confront this metabolic anomaly and its consequences, researchers developed numerous generic and disease specific psychometric tools. With the aid of those psychometric tools the scientific community has started to realize the gruesome effect of diabetes on patients' lives. Diabetic's QoL becomes worse when complications start to develop or comorbidities coexist. Dominant amongst complications, in health-related quality of life (HRQoL) lowering, but not related to risk factors (genetic, the weight of birth, or others) is coronary arterial disease followed by renal failure, blindness, and the combination of micro- and macro-vascular complications and in some studies by sexual dysfunction. Moreover many are the comorbidities which deteriorate further the effect of diabetes in a patient life. Among them obesity, hypertension, dyslipidemia, depression, arthritis are the most common. Most intriguing field for research is the interaction of diabetes and depression and in some cases the progression to dementia. Many aspects and combinations of actions are under researchers' microscope regarding the improvement of HRQoL scores. Until now, the studies performed, have demonstrated little to moderate benefit. More of them are needed to draw safe conclusions on the topic of the best combination of actions to optimize the HRQoL scores.

Key words: Type 2 diabetes; Quality of life; Diabetes co morbidities; Diabetes complications; Dementia; Diabetes type 3

Core tip: Although numerous articles and reviews are written about diabetes every year regarding epidemiology, complications, therapies, comparisons of treatments, health strategies, literature data on diabetic patient's quality of life and how much it is actually affected by complications, comorbidities or different treatments are limited. The current review is focused on: (1) the way patients perceive the changes in different aspects of quality of their lives as recorded by numerous psychometric tools and scales; (2) on the similarities and differences among studies performed worldwide along with the problems and caveats in research; and (3) on aspects intriguing but demanding further research as the effect of diabetes in family life or the common metabolic pathways between diabetes and dementia (recently called also diabetes type 3).

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INTRODUCTION

Diabetes is the increasingly growing metabolic threat of our contemporary era. Diabetes was first described[1] in an Egyptian manuscript from 1500 BC, mentioning "too great emptying of the urine"[2]. Later on, Indian physicians described also the disease and classified it as honey urine by the fact that ants were attacked by patient's urine[2]. The term "diabetes" or "to pass through" was first used in 250 BC by the Greek Apollonius of Memphis[2]. Diabetes type 1 and 2 were recognized for the first time as separate conditions by the Indian physicians Sushruta and Charaka in 400-500 BC, linking type 1 diabetes with youth and type 2 with obesity[2,3]. The term "mellitus" or "from honey" was added by Thomas Willis in the late 1600s because of the sweet taste that urines from diabetic patients had[2]. The first complete clinical description of diabetes was given by the Ancient Greek physician Aretaeus of Cappadocia (1st century AC), who also noted the excessive amount of urine a typical sign of diabetes.

The disease's description has accompanied the human race throughout the centuries. It is found in medieval Persia in Aviccena's *The Canon of Medicine*, in the Roman Empire with Galen describing two cases of diabetic patients during his career[2]. Diabetes was also introduced into Korean and Japanese medicine under the Chinese name táng niào bìng, meaning "sugar urine disease". Although diabetes has been recognized since antiquity, pathogenesis of the disease was understood about 1900[4] while insulin was discovered by Canadians Frederick Banting and Charles Bestthe in 1921 and was first used in 1922[2].

It is well established that the prevalence of diabetes has increased in the developed and developing countries during the last four decades. That is a result of the abundance of food, the consequent change of our dietary habits and the lack of exercise. According to International diabetes Federation, nowadays, one every 11 adults has diabetes (415 million worldwide). By 2040, one adult in 10 (642 million worldwide) will suffer from diabetes. One in 7 births is affected from gestational diabetes and 542000 children worldwide have type 1 diabetes[5]. Additionally every 15 s a person dies from diabetes and the 12% of the global expenditure is spent on diabetes. What is fearful is that 46.5% of adults with diabetes are undiagnosed! In a recent Greek study an age- and sex-adjusted prevalence of diabetes of 10.6% was found, while the prevalence of undiagnosed diabetes was 34%[6].

Progression of diabetes, and especially poor glycemic control, leads to numerous potentially life threatening complications. Almost half of the adults with chronic kidney disease are derived from diabetic population. Likewise, 9.8% of diabetics have experienced heart attack, 9.1% suffer from coronary artery disease (CAD), 7.9% have congestive heart failure, 6.6% have stroke while more than a quarter of them 27.8% suffer from chronic kidney disease, almost a quarter 22.9% have foot problems and last but not least 18.9% have eye damage[4]. All these complications along with the metabolic deterioration demands a large amount of patient's every day energy, planning and thought[7], which leads to a situation called by Rubin[7] "diabetes overwelmus".

QUALITY OF LIFE

The reality is that diabetes influences patients' lives. The mere presence of diabetes deteriorates a person's quality of life (QoL). When diabetes coexists with other chronic illnesses the effect is even worse. But what exactly is QoL? Is it the mere absence of sickness in a man's life? Is it something more? Is it measurable? The worldwide interest is reflected on the 1950000 articles and reviews published the last five years on this research area while the numbers of publications on each diabetic complication are between 15000 and 28000 depending on the complication. Notably only one article was found to asses family functioning.

As Snoek *et al*[8] describes, we are not certain of the origin of the phrase QoL, but American economists Samuel Ordway (1953) and Fairfield Osborn (1954) are considered to be the first to have used the term. Others who used almost the same words was John Galbraith (1967), American president Lyndon B Johnson 1964 followed by social scientists in 1960's who were interested in the new topic of QoL, and particularly the correlation between markers of QoL (such as income level social interaction), and the way individuals perceive them το define their QoL. Surprisingly enough

biological health wasn't a determining factor. Because of the social progress and the medical development, research focused on the issue of well being as patients perceive it.

As Snoek *et al*[8] describes after World War Ⅱ and the introduction of new medicines, the numbers of patients with chronic diseases increased continually. In parallel there was a growing need for evaluation of treatments in terms of medical efficacy but also in terms of everyday life improvement as patients understood it[8]. No sooner than 1976 was the concept of QoL included in the *Index Medicus*[8]. By the year 2000, there had been over 300 articles on the issue of QoL in diabetic population.

In 1997, the World Health Organization (WHO) introduced the first definition of health as "A state of complete physical, mental, and social well-being not merely the absence of disease". WHO, furthermore, introduced QoL as an estimation of well-being as well as a the measurement of health and the effects of health care[9]. WHO defined QoL as individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns. Therefore, except for person's physical health definition of QoL includes psychological state, level of person's independence, social life and personal beliefs[9].

According to United States Centers for Disease Control and Prevention (CDC) QoL is a multidimensional concept that includes evaluations of both positive and negative aspects of a person's life. Since the 1980s, the term health-related quality of life (HRQoL) has comprised those aspects of QoL that can be shown to affect physical or mental health[10-13]. HRQoL includes physical and mental health perceptions (health conditions, social and socioeconomic status) and community-level resources, conditions (practices that influence health perceptions and functional status). According to the above, CDC has defined HRQoL as "an individual's or group's perceived physical and mental health over time"[10-13].

Undoubtedly the answer to the context of happiness and QoL is obscure and although there is no consensus among scientists, it is mostly agreed that QoL: (1) includes many different aspects as mentioned previously; and (2) should be measured through patients perception of well being or the lack of it in their lives[8]. Directly related and a crucial component of QoL is HRQoL. Many times the two concepts have been confused or thought to be identical, or synonymous to well-being, which of course is a mistake. During the last decades the researcher's interest has turned to the concept of disease specific QoL as a treatment goal[14] and important component of therapy. The whole philosophy of diabetes treatment has changed from physician-centered to patient-centered. The last ADA-EASD guidelines focus on patient participation in treatment options along with the physician. Concurrently HRQoL questionnaires have become important component of public health and are considered valid indicators of intervention outcomes and a powerful predictor of mortality and morbidity[15-18].

PSYCHOMETRIC TOOLS

Consequently, the necessity of developing special psychometric tools to measure HRQoL has risen rapidly. Thus, numerous such tools were developed, other generics and other disease specific in an attempt to determine the impact of diabetes and other chronic diseases, along with their complications on patient's lives and also the effect of medical interventions to the evolution of maladies. But as Snoek *et al*[8] states "there isn't a gold standard for the assessment of overall health related or diabetes specific QoL and efforts should be made towards the development of valid, reliable and user friendly assessment tools".

There are many psychometric tools developed in different languages which attempt to asses may aspects of diabetes interference with a person's life. The most used of the later or those which present a special interest are presented below.

The Diabetes Quality of Life Measure (DQOL) was introduced in the Diabetes Control and Complications Trial[19,20]. The scope was to assess four dimensions of diabetes impact: Satisfaction, treatment impact, anxiety for complications and social issues. The DQOL is widely used despite its limitations. Lower scores in this scale are associated with diabetic complications and glycemic control[19,21].

The Diabetes-Specific Quality of Life Scale (DSQOLS) has 64 questions has six dimensions: Social relations, leisure time restrictions and flexibility, physical complaints, worries about the future, diet restriction, and daily hassles. It is used only for type 1 diabetes and it is not validated in English[19,22].

The Diabetes Quality of Life Clinical Trial Questionnaire-Revised has 57 questions measuring physical function, energy, health distress, mental health, satisfaction, treatment satisfaction, treatment flexibility, and frequency of symptoms[19,23].

The Appraisal of Diabetes Scale has 7 questions focusing on diabetic patients' feelings and attitudes and the psychological effect of diabetes[19,24].

The ATT-39 and the revised ATT19 scale focus on the psychological adjustment to diabetes and diabetes integration which is not necessarily synonymous to diabetes specific HRQoL[19,25].

The Questionnaire on Stress in Patients with Diabetes-Revised has 8 dimensions: Leisure and work time, relationship with partner, with doctor, hypoglycemia, therapy, physical symptoms and anxiety about diabetic complications[19,26].

The Type 2 Diabetes Symptom Checklist is a 34-item scale assessing symptoms as hypoglycemic, cardiac, neuropathic, psychological, and vision-related. The scale covers a broad spectrum of symptoms which nevertheless can't always be attributed to diabetes.

The scale was developed in Dutch but there is English translation and validation[19,27].

The Problem Areas in Diabetes Scale (PAID-1) and the revised (PAID-2) are focusing on four dimensions: Overall emotional, interpersonal, treatment-related, and physician-related distress[19,28-30].

The Audit of Diabetes-Dependent Quality of Life (ADDQoL) has 15 questions measuring 13 life domains: Career, social life, family, friendships, sex life, leisure time opportunities, traveling, worries about the future, worries about the future for one's family and friends, and motivation to achieve things[19,31].

The widely used SF36 has 36 questions: An 8-scale profile of biological health and well-being scores as well as psychometrically-based physical and mental health measures and a preference-based health utility index. Physical function, pain, general and mental health, emotional and social function are assessed[32].

DIABETES AND HEALTH RELATED QOL

It is well-known that diabetes *per se*[33] causes a serious deterioration in general QoL mainly affecting the HRQoL. The outcomes are similar worldwide, varying in the grade of influence. Most importantly there are studies[34] implementing that the low QoL anxiety and depression of individuals who, aren't yet officially diagnosed for diabetes but who are at high risk for diabetes. Therefore, clinicians should be educated that high-risk patients at a prediabetic state might have decreased HRQoL and depression, a health dimension that should not be ignored[34].

As shown in a study in three different states in Malaysia there was a statistically important difference in QoL among the three studied populations Malaysian, Indian and Chinese[35]. The Chinese scored significantly lower (21.0 ± 4.3) in the Asian DQOL compared to Malays (81.4 ± 9.0) and Indians (81.5 ± 9.2). Moreover, Chinese scored significantly lower (21.0 ± 4.3) on the Asian DQOL (diet) score compared to Malays (22.8 ± 3.6) and Indians (22.5 ± 3.7). The only component different in a deeper analysis was the different perception of diet among ethnic groups[35]. In the same study, sexual dysfunction lead consistently to lower QoL (-10% in English speaking -5.9%, in Mandarin speaking Chinese, -6% in Malaysians traditional language speaking) in all sub groups whilst there were differences in other predictors. These findings are similar to a Singapore study by Wee *et al*[36] in 2005 which showed ethnicity as an important factor influencing QOL in people with diabetes[37].

In contrast to other studies, the surveys conducted in Nordic population[38] in primary health showed difference between impaired glucose tolerance and overt diabetics whilst the outcomes on HRQoL showed lower scores especially for type 2 diabetics in accordance with literature[39,40]. Older and poorer controlled patients showed lower scores. The most important factor in Nordic studies[38] for the deterioration of HRQoL was the presence of complications, especially CAD and non-vascular complication such as minor psychiatric disorders or musculoskeletal disorders. Nevertheless Viinamäki *et al*[41], found no increased rate of minor mental disorders among diabetic patients but when they coexisted the symptoms tended to be more severe. Furthermore, neuropathy was found to be a predictor of mental disorders in that study. Surprisingly microvascular complications did not have great effect in HRQoL. Other notable findings were that personalization and tailor suited therapy along with continuity in care[42-44] have promising results.

In a study[45], which started in the Cost of Diabetes Type 2 in Europe - (CODE-2) study, a Dutch population of 1371 type 2 diabetics was evaluated using EQ-5D and EuroQol VAS scores for HRQoL and Diabetes Treatment Satisfaction Questionnaire (DTSQ). The outcomes showed good correlation between EQ-5D and EuroQol Vas score although scores in one did not necessarily mean same scores in other. Lower scores were reported as age preceded more, in female sex, with obesity, with insulin use and as complications appeared. Especially low scores were observed for the combination of microvascular and macrovascular complications[45]. Notable points were that anxiety and depression increased and then decreased with age. An explanation given from the writers is that older people attribute their limitations to aging and cope or accept them better than younger people. Another explanation is that in younger populations the fear of future complications is greater. One more interesting point is that duration of diabetes isn't correlating with HRQoL as does not treatment satisfaction. The later is associated with the physician attitude towards the patient and the level of communication between them, fact consistent with literature[14]. The individuals with diabetic neuropathy had lower scores than those with foot ulcers. At last, questions were posed in term of EQ-5D responsiveness to change[45,46].

In another cross sectional study[47], conducted in United States, Self-Administered Quality of Well Being index (QWB-SA) was given to 2048 diabetics type 1 and type 2. Health scores were lower in women and obese patients, and in subjects with kidney disease and arterial hypertension. Scores were substantially lower in type 1 diabetic subjects with retinopathy, neuropathy, foot ulcers, amputation, stroke, and congestive heart failure. The highest scores among the subgroups had the group of diet controlled no obese diabetic men without microvascular, neuropathic, or cardiovascular complications. The same findings were observed in type 2 diabetics. At last, the authors implemented that there might be a correlation between lower than high school education and a deterioration of scores but the writers explained that the sample was inappropriate less than 7% and the chose not to comment on that variable.

Also similar were the findings of a study of diabetic population of a small isolated rural Canadian diabetic population in Bella Coola valley[48]. SF36 and BRFSS

(devised by the CDC, which aims to healthy/unhealthy days and limitations) were used and the scores were correlated to clinic chart information. Of note 57% of diabetic responded, whilst only 37% of non-diabetics. The sample was estimated as representative of the population of diabetics of the area and also in terms of complications CAD (16% *vs* 19%), retinopathy (15% *vs* 14%), cerebrovascular disease (9% *vs* 8%), neuropathy (9% *vs* 10%), peripheral vascular disease (7% *vs* 7%), and nephropathy (6% *vs* 7%). HRQoL scores were lower for diabetics. Factors related to health related QoL scores were duration of diabetes, insulin, and long-term complications of diabetes. Low HbA1c levels were paradoxically associated with lower QoL scores and there was an inverse relationship between duration of diabetes and QoL. The later is consistent with some studies[49] reporting the same outcomes while there are others reporting improvement with age[49-51].

Interestingly there were similar results in a recent review of the Iranian studies[52]. On the topic of the QoL in diabetic population, mostly type 2 and to smaller extend type 1diabetics. Women and older people had lower HRQoL than men and socioeconomic and marital status was positively associated with HRQoL. There were negative associations between HbA1c, BMI, blood pressure, lipids and HRQoL. Also deterioration of HRQoL was shown in the smokers group, whilst conflicting were the results concerning the duration of diabetes and the comparison of rural urban population. The writers note the methodological defaults of the studies. Nevertheless it is notable that the outcomes are consequent with the international studies although there is a difference in culture, diet and exercise habits.

In the UKPDS 37[53], study type 2 diabetics without any complication had a mean EQ-5D index value of 0.83, compared with 0.85 in a Norwegian study[54] conducted by mail in 2006. In the UKPDS 37 study the EQ-5D detected significant differences between people with and without complications In the UKPDS 37 study the EQ-5D detected significant differences between people with and without macrovascular complications, but not microvascular complications. In the same line, in a Singapore cross sectional study by Quah *et al*[55], used EQ5D and SF36 on 699 diabetics reported lower HRQoL in patients with symptomatic complications. This is consistent with many studies[53,56,57].

DIABETES COMPLICATION AND COMORBIDITIES

As seen in the referred above studies diabetes exercises its dark influence when complications start to make their presence in patients' lives. In a Chinese study involving type 2 diabetics[37], which was part of the JADE program Zhang *et al*[37], reported a mean EQ-5D index was 0.897 ± 0.173. Over 80% of diabetics had either hypertension or dyslipidemia and over half were obese. Nephropathy, neuropathy and CAD were associated with low EQ-5D index while retinopathy was not. Notably, hypertension was correlated with EQ-5D index. The outcomes were consistent with a Singapore study[58] while Dutch and Norwegian studies involving Caucasian populations[45,54] reported lower scores. In another Chinese study by Goh *et al*[35], in multiethnic environment diabetic complications had a great impact on QoL.

In the Norwegian study by Solli *et al*[54], patients with complications had reduced HRQoL; 0.90 for those with type 1 diabetes and 0.85 for those with type 2. Presence of one complication decreased scores to 0.76 and 0.80, respectively while with 2 or more diabetic complications the scores were 0.55 and 0.64, respectively. Cerebrovascular disease and neuropathy had a negative impact on overall HRQoL in both types of diabetes, while CAD had an impact on those with type 1 diabetes.

In the Dutch study by Redekop *et al*[45] in type 2 diabetics older patients, female subjects, treatment with insulin, obesity and presence of complications were correlated with a lower HRQoL. In the Canadian Bella Coola survey[48] the rates for diabetes complications regarding CAD (16%), retinopathy (15%), cerebrovascular accidents (9%), neuropathy (9%), peripheral vascular disease (7%), and nephropathy (6%). SF36 scores for diabetics were lower as follows: Physical functioning -13.7, in Social functioning -8.8, in bodily pain -11.1, in role physical -27.4 in role emotional -22, in mental health -3.5 in vitality -6.3, in general health -16.3. Diabetics had more unhealthy days when measured with Mean healthy/unhealthy day scores: +4.4 for unhealthy physical, +2.3 for unhealthy mental, +3.4 for limited by health, +5.4 for limited by pain, +1.9 for felt depressed, +3 for felt anxious, +2.6 for poor sleep, -1.3 for felt healthy. In an American study by Coffey *et al*[47], with 2048 type 1 and 2 diabetics. scores were lower (0.058-0.208) in type 1 diabetics with retinopathy, neuropathy, foot ulcers, amputation, stroke, and congestive heart failure. Health scores were significantly lower (0.052-0.170) in type 2 diabetics with retinopathy, end-stage kidney disease diabetic foot, neuropathy, stroke and heart failure[47]. Ragnarson Tennvall *et al*[59] also, assessed scores in subjects with diabetic foot problems using the EuroQol-EQ5D questionnaire. In this subgroup, major amputations (EQ5D: 0.31) and current foot ulcers (EQ5D: 0.44) were related with lower scores than primary healed ulcers (EQ5D: 0.60) or minor amputations (EQ5D: 0.61).

A Greek study[6] of elderly people living in rural place showed that the most important predictors of impaired HRQoL were female gender (55.4 in the SF36 psychometric tool), diabetic complications, comorbidities and diabetes duration. Older age (56.5 in the SF36 psychometric tool), lower education (60.5 in the SF36 psychometric tool), being unmarried (59.6 in the SF36 psychometric tool), obesity (60.5 in the SF36

psychometric tool), hypertension (62.7 in the SF36 psychometric tool) and dyslipidemia (58.8 in the SF36 psychometric tool) were also associated with impaired HRQoL. In an article of 2006 Piette *et al*[60] note: Most adults with diabetes have at least one comorbid chronic disease and as many as 40% have at least three. The authors categorize comorbidities into groups according to their clinical severity (end stage cancer or stage Ⅳ heart failure), the presence or absence of symptoms (dyslipidemia, hypertension *vs* rheumatoid arthritis) and their concordance or discordance to diabetes (dyslipidemia *vs* low back pain) without clearing the importance of the presence of comorbidities of each category to the evolution of diabetes. In other studies the coexistence of comorbidities resulted in lower scale scores. Also, lower HRQoL was reported in many studies assessing the co-existence of diabetes and other chronic diseases and co morbidities. In a study by Maddigan *et al*[61], the estimated score of diabetics with no complications was slightly lower than the general population, but when co morbidities added up in a patient's life the score deteriorated severely. Triplets of comorbidities were associated with HRQoL deficits. There are studies that correlate exercise with QoL reporting the highest level of physical activity in respondents with better HRQoL and overall health[55]. Wee *et al*[62] describes three possible types of correlation between diabetes and other medical conditions: (1) additive; (2) synergistic; and (3) subtractive relationship, while in his study reports the above mentioned correlation to be additive. He also reports diabetes in general as having moderate influence on subjects in comparison with other chronic illnesses. Another increasingly interesting but not so illuminated point is the interaction between comorbid chronic diseases, innovative treatments such as immunosuppressive agents and the development of overt diabetes to prior non diabetic patients as Pereiraa *et al*[63] high lightened. In this article the authors showed that CsA, tacrolimus and especially rapamycin affected lipolysis of human adipocytes through multiple metabolic pathways and regulations (IL6, TNF, inhibition of mTORC1 and 2 and consequent anomaly in the expression an stimulation of PPARγ) thus impairing the capacity of adipose tissue for plasma lipid clearance, which might contributes to dyslipidemia, fatty liver and promotes the onset of overt diabetes[63].

DEPRESSION, DEMENTIA AND DIABETES: AN INTERESTING TRIANGLE

The coexistence of depression and diabetes has drawn researchers' attention. The fact is quite justified since numerous studies have demonstrated the obscure effect of depression in the evolution of diabetes especially when comorbidities or complications exist[64-66]. Another less studied aspect is the effect of antidepressants on glucose metabolism. Some of them have shown diabetogenic action in non-diabetic depressed patients while others have proved to ameliorate glucose metabolism and consequently they are preferable for treatment of the diabetic population[67]. It is described that effective treatment with antidepressants improves glucose levels in nondiabetics. Cognitive behavioral therapy and selective serotonin reuptake inhibitor (SSRI) improve glycemic control, whereas noradrenergic antidepressants and tricyclic antidepressants cause alter metabolic control[67]. Further illumination on the extremely complex issue of interaction between depression treatment and the development and evolution of diabetes is derived from study of Köhler *et al*[68] who reports a beneficial outcome when statins (most of which is diabetogenic and a standard treatment of diabetic dyslipidemia) are added to SSRIS. The study of Goldney *et al*[64], showed increased prevalence of depression almost 24% of the diabetics compared with 17.1% of the non-diabetics. Also Gavard *et al*[69], in a systematic review of depression in diabetes provided the range of 8.5%-27.3% regarding the prevalence of depression in diabetics. On the other hand depression is related with a 60% increased risk of type 2 diabetes[70]. Goldney *et al*[64], gave an explanation through deterioration of recovery after a cardiac[71], malignancy survival, and predisposition to infection. Many pathways have been proposed for this dysfunctional immune system. The impact of depression on diet, exercise, smoking, alcohol abuse, compliance to treatment regimen. Regardless of the mechanism, the outcomes are clear about the negative role of depression on the course of the diabetes progression[66]. At last Lin *et al*[72], implements those patients with diabetes and coexisting depression are at increased risk. Some of those are infections, dementia, chronic obstructive pulmonary disease and arthritis. The new information emerging from the literature is the characterization of Alzheimer's disease as type 3 diabetes due to common metabolic paths, resistance to insulin and to similar deficits of brain nerve cell along with the improvement of brain cognitive function after intranasal insulin or peroxisome proliferator-activated receptor agonists[73-84]. Similar potential was demonstrated with incretin based therapies[85]. Needless to refer to what dementia does not only to a diabetic's QoL but life itself. To make it worse let us include what dementia does not only to the patient but also to spouses or family's life and QoL.

SOCIAL FUNCTION AND HRQOL

Apart from physical function mental and cognitive decline another aspect of diabetes gruesome influence on HRQoL takes place through the disintegration of the family. In a study by Takenaka *et al*[86], it was demonstrated that family issues were common among type 2 diabetics. The diabetic interacts with the family environment and social net (friends, relatives and acquaintances). Sometimes family acts like diabetes police and other times family doesn't want to participate to patients struggle for better glycemic control. Even

worse, they undermine patient's efforts. The patient reacts with aggressiveness, alienation, spite, or denial to comply, all of which leads to loss of social support, loss of belief in self-efficacy, poorer glycemic control, depression smoking, alcohol use and abuse, consequently complications and comorbidities and dramatic deterioration of HRQoL[73,84,87].

CAN DIABETES HRQOL BE IMPROVED?

Having processed all the above the international community is in search of the proper intervention for the fitting patient and specific divergence. Many studies have been performed and more of them are needed. It is well known the correlation between lifestyle interventions and better glycemic control, hypertension management and lipid management. There are many studies to confirm it[88,89]. In an analysis of 2004 by Ranji *et al*[90], many types of interventions are recognized: (1) provider reminders; (2) facilitated relay of clinical data to providers; (3) audit and feedback; (4) provider education; (5) patient education; (6) promotion of self-management; (7) patient reminders; (8) organizational change; and (9) financial, regulatory, or legislative incentives.

In the same analysis the writers identified as most common type of QoL intervention category of organizational change, followed by patient education and provider education. Moreover, it reports benefit of multifaceted interventions of disease management to a lesser extent and a no statistically significant benefit from the existence of a clinical information system.

Another review by Ricci-Cabello *et al*[91], aimed at quality of care of African Americans reported that interventions targeting self-management, education, reduced the percentage of HbA1c by 0.8%. No such relation was observed with interventions aiming at health care systems and multiple-target interventions

Whilst in a study of Wong *et al*[92], assessing the effect of education interventions among type 2 diabetics showed that there were no associations between the number of sessions attended and HRQoL. Another study evaluated HRQoL in overweight diabetic individuals after attending a weigh-lowering program. The evidence was that the diabetics had significant benefit especially those with the highest baseline BMI and the lower baseline scores[93].

At last a Norwegian review regarding diabetes interventions Sørensen *et al*[89], marked the need for multicomponent interventions targeting patients, health care professionals and policy makers. However, in the same review emphasize the fact that methods to assess the population based impact of these programs in the real world are limited. On the other hand, the outcomes of the Continuous Quality Improvement programme in Catalonia are encouraging and promising since they show the possibilities and potentials of health care interventions on diabetes HRQoL[94].

CONCLUSION

Diabetes continues to be a major contemporary epidemic. In addressing the challenges of confronting the epidemic a primary therapeutic goal is QoL. There is still a lot of confusion regarding the context of QoL, HRQoL and diabetes specific QoL. Recently numerous psychometric tools have been developed in the effort of evaluating QoL, HRQoL and Diabetes specific QoL. Diabetes affects major components of QoL although differences in terms of ethnicity, environment, gender socioeconomic status, culture, profession dietary and lifestyle habits do exist. More specifically: (1) the physical component especially with coexisting obesity complications as CAD renal failure, diabetic neuropathy or retinopathy or co morbidities; (2) the psychological component especially type 1 in younger subjects and in coexistence with depression; (3) the social component by destroying family ties and friendships; and (4) the mental cognitive component particularly when dementia presents.

In that scope numerous worldwide studies have been performed and have demonstrated little to moderate benefit in different components. Towards positive direction is the development of projects such as diabetes quality improvement project but there is al lot to be done in the future[32].

It would be ideal if the same psychometric tools could be translated validated and used in a worldwide scale in order to explore differences in the populations and extract comparable results. At last, diabetes is a strong and cunning enemy demanding all of our resources but technology development and the quality of unexplored yet human brain provide us with the insinuation of a brighter dawn in diabetes homeland.

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MINIREVIEWS

Syndecan-1-coating of interleukin-17-producing natural killer T cells provides a specific method for their visualization and analysis

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Abstract

Natural killer T cells (NKT cells) are innate-like T cells that acquire effector functions while developing in the thymus, polarize into three distinct functional subsets viz. NKT1, NKT2 and NKT17 cells that produce interferon (IFN)-γ, interleukin (IL)-4 and IL-17, respectively. However, there has been no unique surface markers that define each subsets, forcing investigators to use intracellular staining of transcription factors and cytokines in combination of surface markers to distinguish among these subsets. Intracellular staining, however, causes apoptosis and prevents subsequent utilization of NKT cells in functional *in vitro* and *in vivo* assays that require viable cells. This limitation has significantly impeded understanding the specific properties of each subset and their interactions with each other. Therefore, there has been fervent efforts to find a specific markers for each NKT cell subset. We have recently identified that syndecan-1 (SDC-1; CD138) as a specific surface marker of NKT17 cells. This discovery now allows visualization of NKT17 *in situ* and study of their peripheral tissue distribution, characteristics of their TCR and viable sorting for *in vitro* and *in vivo* analysis. In addition, it lays the ground working for investigating significance of SDC-1 expression on this particular subset in regulating their roles in host defense and glucose metabolism.

Key words: Natural killer T cell; NKT17; Syndecan-1 (CD138); Interleukin-17; Body fat

Core tip: Discrete subsets of innate-like Natural killer T (NKT) cells differentially produce three of the most potent and polarizing cytokines, interferon-γ (NKT1), interleukin (IL)-4 (NKT2) and IL-17 (NKT17). But very little is known about how the relationship among the functional subsets of NKT cells is regulated. A major obstacle was the absence of specific single surface markers that reliably identify each subset. Here we

highlight our discovery of syndecan-1 as a specific marker of NKT17 subset and its significance for understanding the role of NKT17 in glucose metabolism and autoimmunity.

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INTRODUCTION

We have recently published that syndecan-1 (SDC-1; CD138) is specifically expressed on the interleukin (IL)-17-producing subset of natural killer cells (NKT17) cells[1]. Briefly, we have previously shown that SDC-1 is expressed on double-negative T cells (DN T cells) that accumulate in lpr and gld mice[2]. After that we sought to know if SDC-1 express in innate cells and detected SDC-1 in a subset of NKT cells. We sorted and analyzed NKT cells subsets by genome-wide gene profiling using microarrays and identified SDC-1 is specifically expressed on the IL-17-producing subset of NKT17 cells[1]. Using SDC-1 expression on NKT17 cells, we visualized their development in the thymus, analyzed their tissue distribution. In addition, we sorted NKT17 cells, out distinguish them from interferon (IFN)-γ-producing NKT1 cells, we sorted each subset and study their characteristics *in vitro*. In this article, we briefly review SDC-1 expression on immune cells and highlight our results and speculate on the potential role of sdc1 in regulating homeostasis of NKT cells and the implication for glucose homeostasis and body fat development.

SDC-1

SDC-1 is a heparan sulfate proteoglycans that is predominantly expressed on epithelial cells[3,4]. It is composed of a short conserved cytoplasmic domain, a transmembrane domain, and a long variable ectodomain carrying heparan sulfate (HS) glycosaminoglycan chains[5]. Sometimes, SDC-1 used chondroitin/dermatan sulfate beside or instead of HS chains[6]. SDC-1 mediates its functions primarily by using HS chains to bind different ligands[7,8]. These include various growth factors such as fibroblast growth factors, Wnt, vascular endothelial growth factor, hepatocyte growth factor, cell matrix proteins, growth factors, cytokines, and chemokines[3,4] and their receptors[9-12]. Ligand binding to HS is regulated at the cell surface by two sulfatases (SULF-1 and SULF-2) and heparanases[13]. Number, position, and orientation of each sulfate group on HS chains play a role in dictating the ability of SDC-1 to bind ligands and initiate downstream signaling events[14-19]. These regulatory sequences have been proposed to act with both autocrine and paracrine mechanisms and represent potential novel targets for therapeutic interventions, particularly against cancer[12]. In addition, recent discoveries indicate that SDC-1 core proteins also has biological functions and can modulate cell behavior independent of HS. In contrast, the transmembrane and cytoplasmic domains of SDC-1 do not have intrinsic kinase or catalytic activity, but yet play important roles in signal transduction pathway by multimerization and/or interaction with other intracellular components, like GTPases or kinases[20]. This often happens in lipid rafts, which are enriched in glycosphingolipids and cholesterol[21] and essential for receptor binding and signal transduction from the cell surface into the cell. In addition, the short cytoplasmic domain of SDC-1 interacts with a number of cytosolic proteins and plays a role in endocytosis.

Using these various mechanisms, SDC-1 regulates multiple cellular functions, including cell proliferation, differentiation, and survival of adherent cells and tumors. Expression of SDC-1 is dysregulated in a number of cancers, including head and neck, ovarian, breast, and colorectal carcinomas[22]. In addition, SDC-1 has been implicated in regulating whole body energy metabolism in Drosophila[23] and body fat in mice[24]. Role of SDC-1 in cancers, infectious diseases, obesity, wound healing, and angiogenesis were reviewed recently[9,22,25] and hence will not be discussed in depth here.

Expression of SDC-1 in immune cells is limited and discrete

While ubiquitously expressed on epithelia and other adherent cells, expression of SDC-1 by the immune cells is limited to few cells as discussed below.

Expression in plasma and B cells: SDC-1 is a well known marker of plasma cells[3] and it has been reported on pre-B cells[26]. Other than that, SDC-1 is not known to be widely expressed among various normal immune cell types. SDC-1, however, is commonly expressed by myeloma cells and lymhopid malignancies and it has been implicated in survival, proliferation and metastasis of tumors[27]. But the exact roles of SDC-1 in the development and function of B cells and plasma cell remain poorly understood.

Specific expression of SDC-1 on NKT17 cells: Invariant NKT cells are highly conserved innate-like T cells that, unlike conventional T cells, are restricted to CD1d molecules and recognize glycolipids as antigens[28]. NKT cells acquire their effector functions while developing in the thymus[29] and differentiate into three distinct subsets that produce IFN-γ, IL-4 or IL-17 cytokine. These subsets were labelled in a manner typical to that of T helper cells (Th)1, (Th)2, and (Th17) cells[29,30]. Hence, the IFN-γ-producing subset is referred to as NKT1, the IL-4 producing subset as NKT2, and the subset that produces IL-17 as the NKT17 subset. Due to their innate nature, NKT cells rapidly produce copius amounts of these cytokines upon stimulation,

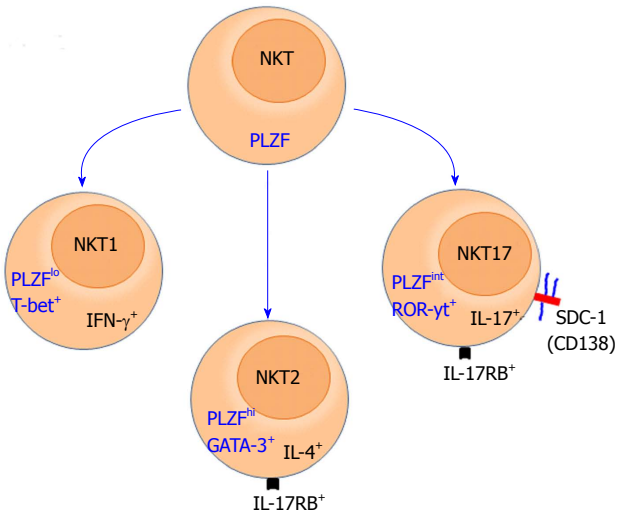

Figure 1 Surface expression of syndecan-1 specifically identifies Natural killer T 17 cells. The three functional subsets are currently distinguished from one another by intracellular staining for specific signature transcription factor or cytokine. Surface expression of SDC-1 can now be used for visualization and sorting of viable NKT17 cells. NKT: Natural killer T; SDC-1: Syndecan-1; IFN: Interferon; IL: Interleukin.

thereby playing critical roles in the initiation and shaping of adaptive immune responses[29,31,32]. These cytokines are highly potent and capable of polarizing adaptive immune responses into Th1, Th2 or Th17 type. Furthermore, because of the ability of these cytokines to inhibit each other function, the overall physiological functions of NKT cells and how the opposing functions of the three subsets are reconciled under physiological and pathological condition remain a mystery. Lack of progress in solving this paradox is rooted in the absence of reliable surface markers that identify and distinguish subsets of NKT cells from one another.

Currently, distinguishing among the NKT subsets is made using intracellular staining for signature transcription factors that control production of IFN-γ (Tbet), IL-4 (PLZF and GATA3), and IL-17 [retinoic acid-related orphan receptor γt (RORγt)][4,33]. Additionally, NKT subsets are identified based on intracellular staining for their signature cytokine, IFN-γ (NKT1), IL-4 (NKT2) and IL-17 (NKT17). Otherwise, It has been difficult to definitively distinguish among NKT cell subsets. Intracellular staining, however, requires fixation and permeabilization, which is a serious limitation that abroagtes the ability of investigators to do *in vitro* functional analysis using purified invidudual subsets. It has also impeded *in vivo* tracking and characterization of individual NKT cell subsets and full appreciation of the pathophysiologic functions of each subset. To avoid this problem, a combination of surface markers are currently used for this purpose, but they have their own shortcomings. For example, NKT17 cells can be identified based on low expression of NK1.1 and CD4, and high expression of CCR6 and IL17RB[34]. However, IL-17RB is also expressed by NKT2 cells and expression of NK1.1 is a strain-dependent and absent in most mouse strains[35]. Our recent identification of SDC-1 as a specific marker of NKT17 cells overcome this challenge at least for this subset[1] (Figure 1). This finding has been confirmed by three independent studies[36-38]. This discovery now allows visualization of NKT17 in the thymus and their peripheral tissue distribution, which is leading to novel insights into NKT cell biology.

Implication of SDC-1 expression on NKT17 cells on host defense and glucose metabolism

IL-17 is a potent proinflammatory cytokines that is required in host defense against infections[39] and been implicated in pathogenesis of asthma[40], and autoimmune diseases such as type 1 diabetes[41-43] and regulation of body fat[44]. In addition, IL-17 has been reported to modulate both adipogenesis and functions of adipocytes and glucose metabolism in mice[44,45]. Both IL-17AKO and IL-17RAKO mice has been reported to gain in weight due to the accumulation of visceral fat[44], suggesting involvement of IL-17 in maintaining body fat. NKT17 cells represent about 20% of NKT cells in the thymus[1] and approximately 2%-10% of total NKT cells in secondary lymphoid organs. NKT17 can secrete large amounts of IL-17 in response to various stimuli, such as infections, allergens, tissue injury and metabolic disorders[46,47].

Interestingly, NKT17 cells preferentially reside in visceral adipose tissue in mice[1] and their local and systemic frequencies are reduced in obese patients, suggesting their involvement in inflammation during obesity[48]. In addition, it has been reported that NKT17 could play a pathogenic role in the pathophysiology of diabetes[41]. Therefore, we speculate that studies addressing the roles of SDC-1 expressing NKT17 cell may provide an alternative approach to understanding its role in fat metabolism and glucose homeostasis. Thus, the findings by our group and subsequently other groups that NKT17 cells are identifiable by surface experssion SDC-1 is crucial for clear understanding of their biology and regulation and their physiologic role in thes steady state and disease condition[1,27,38]. For example, SDC-1 provides a unique opportunity for tracking and analysis of NKT17 cells *in vivo* and for sorting viable NKT17 for various *in vitro* functional studies and adoptive transfer experiments. In this regards, our findings of great responsiveness of NKT17 than do NKT1 cells is consistent with their preferential localization of NKT17 in white adipose tissue (WAT) and suggest special link to WAT.

CONCLUSION

The discovery of SDC-1 as specific marker for NKT17 cells laid the foundation for understanding the biology of NKT17 cells and their pathophysiologic functions. In addition, it will be helpful in uncovering specific markers for NKT1 and NKT2 by excluding NKT17 cells and sorting of pure NKT1 and NKT2 cells for gene expression profiling. Future studies are expected to develop into

understanding the significance of selective expression of SDC-1 by NKT17 cells and generating new information into the role of SDC-1 in the immune cells, which can lead to develoment of new strategies for manipulating individual subsets of NKT cells for therapeutic purposes.

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MINIREVIEWS

Osteomyelitis in diabetic foot: A comprehensive overview

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Abstract

Foot infection is a well recognized risk factor for major amputation in diabetic patients. The osteomyelitis is one of the most common expression of diabetic foot infection, being present approximately in present in 10%-15% of moderate and in 50% of severe infectious process. An early and accurate diagnosis is required to ensure a targeted treatment and reduce the risk of major amputation. The aim of this review is to report a complete overview about the management of diabetic foot osteomyelitis. Epidemiology, clinical aspects, diagnosis and treatment are widely described according to scientific reccomendations and our experience.

Key words: Diabetic foot ulcers; Diabetic foot infections; Osteomyelitis; Surgery; Antibiotic therapy

Core tip: Diabetic foot osteomyelitis is a current topic in the field of diabetic foot. Bone infection is a recognized risk factor for minor and major amputation. An accurate description about the diagnosis and treatment is useful to help physicians in the management of osteomyelitis in patients affected by diabetic foot ulcers.

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INTRODUCTION

Approximately 60% of diabetic foot ulcers (DFUs) are complicated by infection[1]. In more than two-thirds of the cases, infection is the main cause for major lower limb amputation in diabetic patients with foot ulceration[2-5]. Infections may complicate DFUs in both neuropathic and ischemic ulcers.

However, the simultaneous presence of peripheral arterial disease (PAD) and infection influence the evolution of DFUs, increasing the risk of non-healing and major amputation[1]. Therefore, in the case of diabetic foot infection (DFI) and limb ischemia, , it is mandatory an early performing revascularization to allow an

adequate blood flow in the area of the infection.

Even if a large variety of bacteria may colonize foot ulcers, infection is considered only if an inflammatory reaction develops due to the interaction between bacteria and host tissues. Colonization is usually limited to skin surface, while infection is generally characterized by the involvement of subcutaneous or deepest tissues. The severity of infection is related to location, depth (fascia, muscles, tendons, joints or bone), presence of necrosis and/or gangrene.

The diagnosis of infection is usually clinical while the microbiological characterization allows to detect the bacteria involved and drive the targeted antibiotic treatment.

Gram positive bacteria as staphylococcus aureus are the most involved in DFI. Nowadays, the resistance to antibiotics is increasing in diabetic population and multi-resistant organisms (MDRO) are common in DFI. Hospitalization, surgical procedures and long antibiotic therapy induce the development of MDRO or methicillin-resistant Staphylococcus aureus (MRSA)[6-8]. Osteomyelitis is a common DFUs infection, being present in 10%-15% of moderate and in 50% of severe infections[9]. The ulcers complicated by osteomyelitis often require surgical treatments and a long antibiotic therapy too[10-12].

Osteomyelitis is usually due to non-healing ulcers and it is associated with high risk of major amputation[13-15].

Diabetic foot osteomyelitis (DFO) is mostly the consequence of a soft tissue infection that spreads into the bone, involving the cortex first and then the marrow. The possible bone involvement should be suspected in all DFUs patients with infection clinical findings, in chronic wounds and in case of ulcer recurrence.

Osteomyelitis can affect any bone but most frequently the forefoot (90%), followed by the midfoot (5%) and the hindfoot (5%). Forefoot have a better prognosis than midfoot and hindfoot osteomyelitis. Above the ankle amputation risk is significantly higher for hindfoot (50%), than midfoot (18.5%) and forefoot (0.33%)[16-18]. An early and accurate diagnosis is required to ensure an effective treatment and reduce the risk of minor and major amputation[19,20].

THE MICROBIOLOGY OF OSTEOMYELITIS

The microorganisms involved in DFI show a various epidemiology depending on the characteristics of the patient, the clinical risk factors, the wounds (extension and depth) and the microenvironment.

The epidemiology of osteomyelitis reflects the one found in soft tissue infections, rarely mono-microbial and more often poly-microbial. *S. aureus* (up to 50% of cases), *S. epidermidis* (about 25%), *Streptococci* (about 30%) and *Enterobacteriaceae* (up to 40%) are the most commonly detected bacteria in DFO[21,22]. Among the Gram negative, *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus*, are the most common microorganism followed by *Pseudomonas aeruginosa*. The rate of anaerobes is usually low[21,22]. Also DFO show an increased MDRO mainly MRSA or extended-spectrum of beta-lactamase-producing[6-8]. The multi-drug resistance is a current topic for clinicians with significant influence on antibiotic approach.

DIAGNOSIS OF OSTEOMYELITIS

The diagnosis should be first based on clinical signs of infection supported by laboratory, microbiological and radiological evaluation. However, the diagnosis remains a challenge and DFO is often not recognized easily in its initial phase.

Infected wounds usually show purulent secretions or at least two signs of inflammation (swelling, erythema, blood serum secretion or simply blood with or without bone fragments)[14]. However, DFO can occur without any local sign of inflammation. Systemic symptoms such as fever and malaise are rare, especially in case of chronic osteomyelitis.

Various clinical findings can help clinicians in detecting bone infection. Two specific clinical signs are predictive of osteomyelitis. The first is the width and depth of the foot ulcer. An ulcer larger than 2 cm^2 has a sensitivity of 56% and a specificity of 92%. Deep ulcers (> 3 mm) are more easily associated with an underlying osteomyelitis than superficial ulcers (82% *vs* 33%)[23].

A second diagnostic criterion to detect DFO is the "probe-to-bone test" (PTB). PBT is performed probing the ulcer area with a sterile blunt probe. If the probe reaches the bone surface the PTB is considered positive. In a study involving 75 diabetic patients, PTB showed a sensitivity of 66%, a specificity of 85% and a positive predictive value of 89%[24]. The same test, evaluated in a subsequent prospective study of 1666 diabetic patients and compared with the culture of infected bones, was found to have a sensitivity of 87%, a specificity of 91%, a positive predictive value of only 57% and a negative predictive value of 98%[25].

Therefore, in the presence of infected ulcers, a positive PTB test is highly suggestive of osteomyelitis, but a negative test does not exclude it. Instead, in presence of an ulcer without clinical signs of infection, a positive test may be not specific for osteomyelitis while a negative PBT test should exclude a bone infection[26].

The combination of the PTB test with X-ray improve the sensitivity and specificity in the diagnosis of DFO[27,28]. Bone infection is also considered in case of visible or exposed bone or discharge of bone fragments (Figures 1 and 2).

Serum inflammatory markers as white blood cells (WBC), C-reactive protein, erythrocyte sedimentation rate (ESR) and procalcitonin (PCT) are usually higher in DFO than soft-tissue infections. However, WBC and procalctitonin may be negative while ESR > 60 mm/h and/or CRP > 3.2 mg/dL in the presence of an ulcer deeper than > 3 mm are significantly predictive of DFO[29]. Furthermore, WBC, CRP and PCT values return to their normal range approximately in three weeks

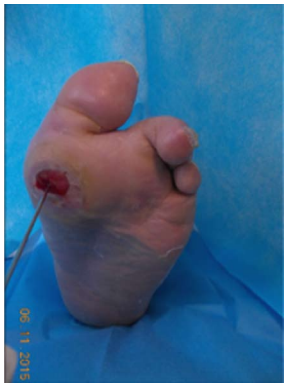

Figure 1 Positive probe-to-bone test for first metatarsal head.

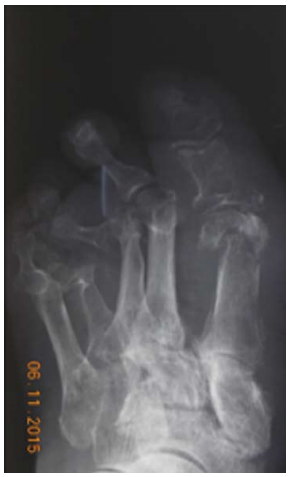

Figure 2 X-ray showing destruction of first metatarsal head.

after the treatment in both of soft-tissue and bone infection, while ESR usually remains high only in case of osteomyelitis[30].

Radiological tests are usually required to detect bone involvement in case of suspect osteomyelitis without clinical signs of infection, to confirm the clinical suspicion and detect the affected bone/bones and to distinguish DFO from soft tissue infection. X-ray is the first instrumental tool although it's arduous to detect the infectious process during the initial phase. Clear signs related to osteomyelitis are generally not evident until 30%-50% of the bone has not been involved; usually this condition happens after 2-3 wk. X-ray DFO imaging are usually characterized by osteopenia, erosion of cortical bone, cortical lysis, osteolysis, periosteal thickening, bone sequestration[31,32]. Radiological criteria of bone healing include: Well-organized consolidation of periosteum, reduction of bone lucency, reduction of pathological fractures related to bone infection, neo-formation of mineralized bone in the areas destroyed by the infection[33].

Scintigraphic examinations are more sensitive than X-ray, especially during the earliest stage of bone infection and the follow-up. However, the common limitation is the low specificity in the discrimination between

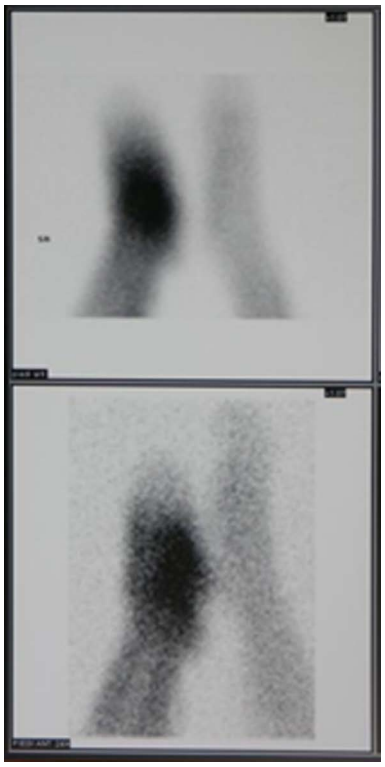

Figure 3 Leucocyte scan images showing area of increased uptake strongly suggestive of osteomyelitis in left mid and hindfoot.

soft tissues and bone infection[34]. The specificity of leucocyte scan is better than triple-phase bone scan even if the spatial resolution can be a limiting factor. However, labeled leukocyte imaging are more useful than bone scan for diagnosis, evaluation of bone affected and follow-up during medical treatment[35,36].

More recently, it has been shown that combined 99mTc white blood cell-labeled single-photon emission computed tomography and computed tomography (99mTc WBC labelled-SPECT/CT) imaging provide good spatial resolution with the three-dimensional CT-scan images and WBC uptake intensity yielding more information about the location and extension of infection[37,38]. Particularly, the role of 99mTc WBC labeled-SPECT/CT has been positively evaluated to identify the complete resolution of infection during the follow-up of patients treated by antibiotics[39] (Figure 3). The positron emission tomography-computed tomography (PET/CT) with fluorine-18-fluorodeoxyglucose (^{18}F-FDG) is an excellent hybrid imaging that can be used in the diagnosis of DFO and to distinguish bone from soft tissues infections. ^{18}F-FDG is a non-specific tracer to evaluate intracellular glucose metabolism; its uptake in increased in the areas of infection and inflammation[40-42] (Figure 4).

Magnetic resonance imaging (MRI) with gadolinium shows very high sensitivity (90%), and specificity (85%) in the diagnosis of DFO. The gadolinium uptake allows to distinguish between soft tissues and bone better than CT and scintigraphic methods[43,44]. The typical

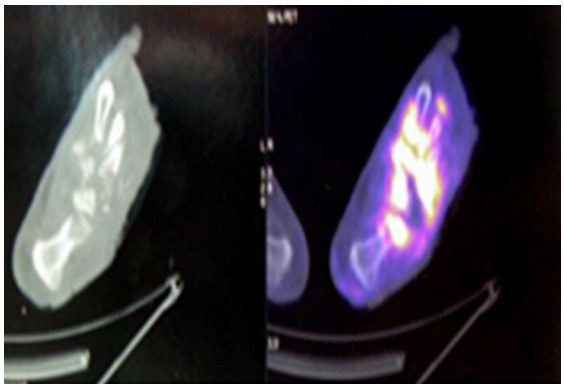

Figure 4 Positron emission tomography images demonstrating diffuse increased of 18F-2-fluoro-2-deoxy-D-glucose uptake of the right foot suggestive of severe osteomyelitis.

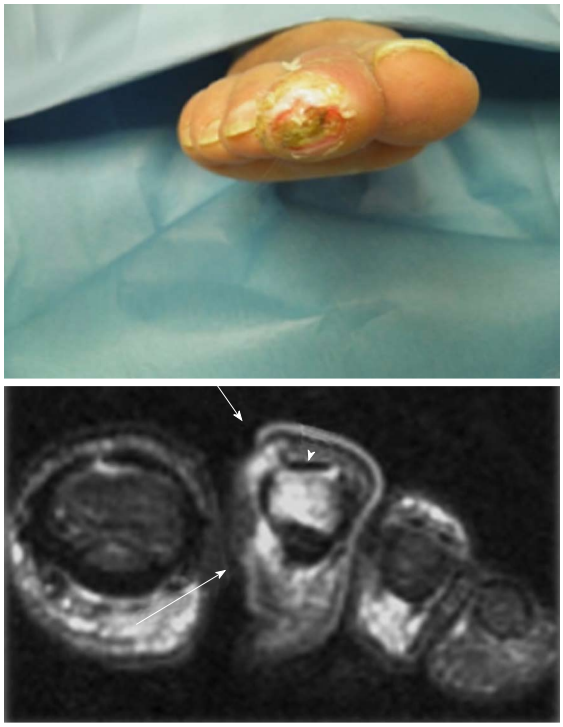

Figure 5 Osteomyelitis of second toe (distal phalanx) revealed by magnetic resonance imaging. The arrows and the arrowhead show the bone involvement of distal phalanx (second toe).

changes in the bone marrow predictive for osteomyelitis are low signal intensity on T1-weighted sequences and high signal intensity on T2-weighted sequences. These findings may be already evident 3 d after the onset of infection. The major limit is the reduced resolution in the evaluation of cortical bone that does not allow to highlight few cases of infection such as osteitis or to distinguish other causes of bone injury[45,46].

The guidelines suggest that the diagnosis of DFO include the combination of different diagnostic tests, such as PTB, serum inflammatory markers, X-ray, MRI or radionuclide scanning. X-ray should be always the first imaging evaluation; when more specific imaging are required, MRI is the first choice while white blood

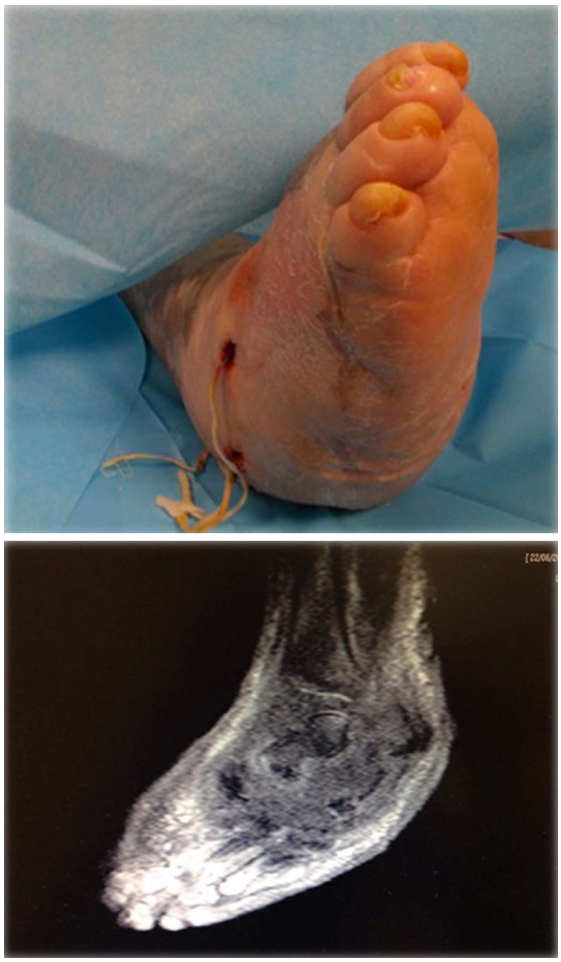

Figure 6 Severe osteomyelitis involving midfoot, hindfoot and ankle detected by magnetic resonance imaging.

cell-labelled radionuclide scan, SPECT/CT and ^{18}F-FDG PET/CT are used only if MRI is contraindicated[21,22] (Figures 5-7).

The gold standard for the diagnosis of osteomyelitis is the bone biopsy which provides histological and microbiological findings[21,22]. Histological criteria are: Bone erosion, marrow edema, fibrosis, necrosis, presence of inflammatory cells (both acute and chronic), seizure. Furthermore, the bone biopsy allows to identify precisely the bacteria involved in the infectious process and to evaluate the susceptibility to antibiotic therapy. The bone can be removed by a percutaneous approach through a not infected skin or during the open surgical procedures. In case of bone infection, superficial swab shows a low sensitivity, in fact a reliable correspondence between bacteria isolated form bone biopsy and swab culture is approximately of 38%[47]. Therefore, superficial swab should not be used in case of DFO. Bone biopsy is the most accurate test (preferably after 10 d of antibiotic suspension) even if in several cases it is not technically feasible. However, a recent study showed that the pathogens isolated from culture of deep tissues (removed from the area closest to the bone) are very similar to those obtained from bone biopsy (74.3% *vs* 82.8%)[48].

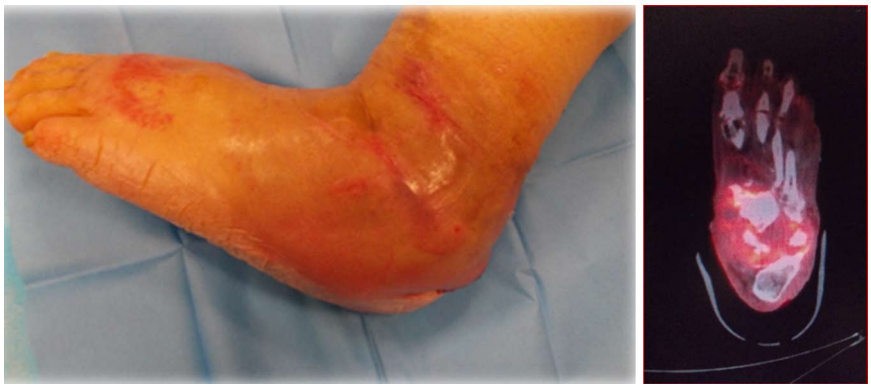

Figure 7 Severe osteomyelitis of forefoot, mid and hindfoot by positron emission tomography-computed tomography.

TREATMENT OF OSTEOMYELITIS

The treatment of DFO remains a hot topic in the field of diabetic foot. Over the years the most debated theories have been surgical or antibiotic therapy as first approach.

Nowadays the treatment of osteomyelitis is not completely standardized and evaluated case by case. Therefore, the guidelines broadly recommend the specific conditions for surgical or medical approach combined with conservative surgery. Conservative surgery means usually a procedure in which only the infected bone and the non-viable soft tissues are removed without any amputation[16].

Tan *et al*[49] have shown that an aggressive surgical approach with minor amputation reduces the risk of major amputation above the ankle and reduce the length of hospitalization and associated costs. The Authors report that forefoot amputation reduces the risk of major amputation in comparison to medical therapy performed for 3 d[49]. However, antibiotic therapy was performed only for 3 d and it is well known that DFO can require long antibiotic therapy.

Although an aggressive surgical approach could be mandatory under some circumstances, retrospective studies have shown that conservative treatment associated with prolonged antibiotic therapy is effective to promote wound healing and reduce the risk of major amputation and of ulcers recurrence[16,50,51].

Ha Van *et al*[50] have compared the conservative surgical treatment, defined as the resection of limited part of infected bone (phalanx and/or metatarsal head), associated with antibiotic therapy against antibiotic therapy alone. The conservative approach was more effective in terms of ulcer healing (78% *vs* 57%) and healing time (181 ± 30 d *vs* 462 ± 98 d, $P < 0.008$) compared to antibiotic therapy alone. Furthermore, the length of antibiotic therapy was significantly reduced in the group treated by conservative approach than the group treated by antibiotic alone (111 ± 121 d *vs* 246.9 ± 232 d, $P < 0.007$)[50].

Antibiotic therapy is widely used in association to surgical approach, both for minimal or extended procedures; however, several studies have reported many cases of DFO treated only by antibiotic therapy without surgery. Some Authors have reduced the role of surgery to treat bone infection, mainly in case of chronic osteomyelitis[52-54].

A recent prospective randomized clinical study has compared conservative surgery (removal of bone without amputation of any part of the foot) and antibiotic therapy alone. Severe infection, patients with PAD and severe co-morbidity were excluded. Osteomyelitis were located in the forefoot. The surgical group received empirical antibiotic therapy after the procedure. The group treated by antibiotics alone received for 90 d a targeted treatment according to the microbiological culture of deep soft tissues localized near the bone. The patient were followed for 12 mo after wound healing The rate of wound healing and healing time for respectively surgical and medical groups was similar (86.3% *vs* 75%) and (6 wk *vs* 7 wk). Only 16.6% of subjects treated by antibiotics alone required a secondary surgical approach. No patient received major amputation[55].

Also the optimal duration of antibiotic therapy is not completely defined. The Infectious Disease Society of America (IDSA) considers 4-6 wk adequate when the infected bone is not completely removed by surgery while at least 3 mo in case of antibiotic therapy alone[21]. However, the recent report of International Working Group of Diabetic Foot (IWGDF) suggested 6 wk of antibiotic therapy if the infected bone was not removed by surgery and no more than a week if infected bone was resected[22]. Lately, the aim is to reduce the duration of antibiotic therapy. In fact, prolonged use of antibiotics increases the risk of bacterial resistance, side effects and costs.

A prospective randomized study compared two groups of not ischemic patients with DFUs on the forefoot complicated by osteomyelitis treated with antibiotic therapy respectively for 6 or 12 wk. At the beginning antibiotic therapy was empirical and then driven by microbiological results. Sixty-six percent of patients resolved the osteomyelitis and there was not a significant difference between the two groups. Furthermore, the group treated for 12 wk showed more

side effects than the group treated for six weeks[56].

A significant aspect is to define the resolution of bone infection. Nowadays, there are no tests correlated to long-term resolution of osteomyelitis. The IWGDF suggest that a decrease of serum inflammatory markers, especially ESR, associated with the resolution of soft tissue infection, healing and positive evolution of radiological signs can be used to stop antibiotic therapy.

Chronic osteomyelitis is associated with a high percentage of recurrence despite a long antibiotic therapy. The rate of infection recurrence is approximately of 30%[31,54]. Recurrence might be related to the incomplete resection of infected bone or to resistant microorganisms persistently remaining in their biofilm[57]. The recurrence of DFO has to be considered in case of ulcer reappearance within 12 mo after the first healing. Furthermore, recurrent foot ulceration can promote the reappearance of bone infection. Adequate prevention is mandatory.

CRITICAL ISSUES

The appropriate management of DFO is closely based on both the severity of infection and patient's characteristics. Surgical and conservative approach shows advantages in some conditions and disadvantages in other. Several factors can influence the outcome. Among the advantages of surgical therapy there is the complete removal of the infected bone and the reduced duration of antibiotic therapy. On the other side an aggressive approach can lead to an extended tissue loss and it should be done only in patients with an adequate blood perfusion.

Further, the surgical treatment can impair the foot balance. In fact, a partial amputation (such as removal of a ray or a metatarsal head), mainly if associated to a pre-existing peripheral neuropathy, can increase biomechanical impairments of the foot and promote re-ulceration or new ulcerations in different areas.

Armstrong *et al*[58] have shown that forefoot amputation (toes or rays) reduces the joint mobility and increases the plantar pressures, 10-fold higher than that found in patients without forefoot amputation. Furthermore, increased peak pressure and limited joint mobility are significantly related high risk of re-amputation.

Molines-Barroso *et al*[59] analyzed the risk factors of re-ulceration in 119 diabetic patients who underwent resection of the metatarsal heads due to osteomyelitis. The rate of re-ulceration was higher in case of 1st and 3rd metatarsal head resection (69% and 52% respectively), followed by the resection of 2nd, 4th and 5th metatarsal head (44%, 25% and 19% respectively). The removal of more than one metatarsal heads was associated with a risk of re-ulceration approximately of 50%. The risk of ulceration transfer was significantly higher in case of 1st metatarsal head resection (P = 0.004)[59]. The main advantages of the medical treatment is to avoid the surgical treatment preserving the foot architecture and biomechanics.

The IDSA guidelines define the four clinical patterns where antibiotic therapy without surgery should be considered: (1) high risk of foot function loss in case of radical resection of infected bone; (2) severe deficiency in foot perfusion without chance of revascularization; (3) infection confined to the forefoot with only a minimal loss of soft tissue; and (4) excessive surgical risk according to patients general conditions. Furthermore, antibiotic therapy should be the first choice in case of small ulcers of the forefoot without bone exposure.

The main disadvantages of medical therapy may be the increased risk of infection recurrence, the long duration that can predispose to side effects and promote antibiotic resistance. According to IWGDF guidance, surgical bone resection is recommended in cases of bone exposure, progressive bone destruction and spreading of infection along the soft tissues[22].

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ORIGINAL ARTICLE

Basic Study

Insulin-mimetic compound hexaquis (benzylammonium) decavanadate is antilipolytic in human fat cells

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Abstract

AIM

To assess in rodent and human adipocytes the antilipolytic capacity of hexaquis(benzylammonium) decavanadate (B6V10), previously shown to exert antidiabetic effects in rodent models, such as lowering free fatty acids (FFA) and glucose circulating levels.

METHODS

Adipose tissue (AT) samples were obtained after informed consent from overweight women undergoing plastic surgery. Comparison of the effects of B6V10 and reference antilipolytic agents (insulin, benzylamine, vanadate) on the lipolytic activity was performed on adipocytes freshly isolated from rat, mouse and human AT. Glycerol release was measured using colorimetric assay as an index of lipolytic activity. The influence of B6V10 and reference agents on glucose transport into human fat cells was determined using the radiolabelled 2-deoxyglucose uptake assay.

RESULTS

In all the species studied, B6V10 exhibited a dose-dependent inhibition of adipocyte lipolysis when triglyceride breakdown was moderately enhanced by β-adrenergic receptor stimulation. B6V10 exerted on human adipocyte a maximal lipolysis inhibition of glycerol release that was stronger than that elicited by insulin. However, B6V10 did not inhibit basal and maximally stimulated lipolysis. When incubated at dose $\geqslant$ 10 μmol/L, B6V10 stimulated by twofold the glucose uptake in human fat cells, but - similarly to benzylamine - without reaching the maximal effect of insulin, while it reproduced one-half of the insulin-stimulation of lipogenesis in mouse fat cells.

CONCLUSION

B6V10 exerts insulin-like actions in adipocytes, including lipolysis inhibition and glucose transport activation. B6V10 may be useful in limiting lipotoxicity related to obesity and insulin resistance.

Key words: Adipocyte; Lipolysis; Amine oxidases; Insulin resistance; Obesity; Hydrogen peroxide; Vanadium; Antidiabetics

Core tip: This study investigates in murine and human adipocytes the antilipolytic properties of a conjugate of benzylamine and decavanadate (B6V10), already reported to lower hyperglycaemia in diabetic rodents. Data indicated that the conjugate dose-dependently inhibited submaximal activation of lipolysis in all the species studied. Such antilipolytic action deals with the *in vivo* FFA-lowering properties already described for B6V10 in diabetic rats. B6V10 also activated lipogenesis and glucose transport in fat cells. B6V10 should therefore be useful in preventing the lipotoxicity constituted by the unrestrained lipolytic activity of insulin-resistant adipocytes in obese individuals presenting type 2 diabetes, a state named diabesity.

Carpéné C, Garcia-Vicente S, Serrano M, Marti L, Belles C, Royo M, Galitzky J, Zorzano A, Testar X. Insulin-mimetic compound hexaquis (benzylammonium) decavanadate is antilipolytic in human fat cells. *World J Diabetes* 2017; 8(4): 143-153 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i4/143.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i4.143

INTRODUCTION

In obesity, the excessive enlargement of the adipose tissue (AT) is often associated with type 2 diabetes and morbid complications, especially when the hypertrophied fat depots are located in the intra-abdominal cavity (known as visceral fat). More than a decade ago, the links between fatness and altered glucose and lipid handling led to propose the term diabesity to define a complex disease distinct from "healthy" obesity[1]. The function of AT is not restricted to lipid storage: Indeed, it is also an endocrine organ, secreting numerous adipokines. Therefore, the excess of AT can be associated with insulin resistance[2,3], endocrine, metabolic and inflammatory disturbances, increasing the risk of co-morbidities, such as hypertension and dyslipidaemia. However, all these disorders, known as metabolic syndrome[4], not only co-exist with hypertrophied lipid storage but also with excessive lipid mobilization since the entire lipid turnover is dysregulated in diabesity. In fact, the circulating levels of the products of adipocyte lipolysis, namely free fatty acids (FFA) and glycerol, are dramatically elevated in obese individuals[5]. Such increase is likely resulting from a defective responsiveness of the adipocytes to the antilipolytic action of insulin. It is important to mention that insulin not only stimulates triglyceride synthesis, but also inhibits triglyceride breakdown, lowering basal lipolysis in fat cells and reducing FFA and glycerol blood levels. Therefore, in obese subjects with insulin resistance, the hypertrophied adipocytes release excessive amounts of FFA, which are not a good fuel supply to the other organs, and even hamper carbohydrate utilization. This contributes to maintaining

insulin resistance and its deleterious outcomes. Especially when occurring in visceral AT, such excessive lipolysis results in a high flux of FFA toward the liver, causing hepatosteatosis, inflammation, and worsening dyslipidemia. It is admitted that subjects with visceral fat have higher postprandial FFA and are at a higher risk of fatty liver disease and hepatic insulin resistance[6-8]. Indeed, clinical studies have demonstrated that the insulin resistance occurring in excessive AT affects metabolic parameters and increases liver damage[9]. Excessive FFA also have toxic effects in other organs (*e.g.,* alteration of insulin secretion in pancreas[10]), that contribute to exacerbate hyperinsulinemia and insulin resistance. At the cellular level, excessive FFA supply impairs mitochondrial function and leads to abnormal lipid oxidation, further disturbing lipid turnover and cell survival. All these effects of excessive FFA belong to a network of mechanisms currently defined as lipotoxicity[11].

Since unrestrained AT lipolysis results in increased fatty acid release, leading to lipotoxicity, the search for antilipolytic drugs has been re-considered recently as a promising approach to delay and/or reverse the onset of insulin resistance in diabesity. Consequently, many pharmacological agents are under investigation with the objective of reproducing and surpassing the beneficial effects of the classical antilipolytic agent Acipimox, reported to transiently alleviate insulin resistance in obese subjects[12]. Agonists of Gi-protein coupled receptors endowed with such antilipolytic properties have been reviewed elsewhere[5]. In this context, we aimed to verify in adipocytes the antilipolytic properties of a potential antidiabetic agent previously characterized as an insulin-mimicker on its basis to activate glucose transport in adipocytes from rodent models[13].

Our interest was therefore focused in searching how an arylalkylamine vanadium salt, endowed with insulin-like actions regarding glucose disposal[14], was able to directly reduce the lipolytic activity of freshly isolated adipocytes. Our previous studies showed that hexaquis(benzylammonium) decavanadate, the formula of which is $(C_7H_{10}N)_6V_{10}O_{28}.2H_2O$, is a salt conjugate of benzylamine and decavanate (B6V10) acting as a substrate for semicarbazide sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1)[15]. This enzyme is abundant at the surface of adipocytes and generates hydrogen peroxide when oxidizing its amine substrates. In the presence of B6V10, SSAO/VAP-1 also generated substantial amount of peroxovanadium, which, *via* phosphatase inhibition, was able to trigger insulin signalling downstream of the insulin receptor and to activate glucose transport in rodent adipocytes in the complete absence of insulin[16]. Chronic administration of B6V10 to rat or mouse models of diabetes substantially lowered blood glucose levels[13]. In addition, B6V10 normalized the plasma concentration of non-esterified fatty acids in severely diabetic rats[13]. Our present study consisted in a comparative approach testing under various conditions the putative antilipolytic actions of B6V10 in murine and human adipocytes.

We first tested increasing doses of B6V10 (0.1 to 100 μmol/L) on the triglyceride breakdown (lipolysis releasing FFA and glycerol) in rat adipocytes. Then a broader range of B6V10 doses (1 nmol/L to 100 μmol/L) was tested on the lipolytic and lipogenic responses of mouse adipocytes. Finally, our observations showed for the first time in human adipocytes a substantial antilipolytic action of supramicromolar doses of B6V10, which also activated glucose uptake.

MATERIALS AND METHODS

Patients and human adipocyte preparations

Adipocytes were isolated from samples of subcutaneous adipose tissue obtained from women undergoing abdominal lipectomy under the control of plastic surgery staff of Rangueil Hospital (Toulouse, France). A total of 13 overweight women (age range: 30-48 year, BMI = 25.9 ± 1.1 kg/m^2) were incorporated in the study following agreement of the INSERM guidelines and local ethic committee. The surgically removed pieces of human adipose tissue were placed in sterile plastic box, and transferred in less than one hour to the laboratory. The samples were immediately subjected to collagenase digestion at 37 ℃ to obtain freshly isolated adipose cells. To do so, pieces of adipose tissue were minced with scissors in Krebs-Ringer salt solution pH 7.5 containing 15 mmol/L sodium bicarbonate, 10 mmol/L HEPES and 3.5% of fat-depleted bovine serum albumin (KRBHA), and 5.5 mmol/L glucose. For the cell preparations used for glucose uptake assays, glucose was replaced by 2 mmol/L pyruvate. After digestion with 1 mg/mL collagenase type Ⅱ for approximately 45 min under agitation, buoyant adipocytes were separated by filtration through a 300 μmol/L mesh-screen and carefully washed in fresh medium to obtain adipocyte suspensions as previously described[17]. Final adipocyte suspensions averaged 14.5 ± 1.4 mg cell lipids/400 μL unless otherwise stated.

Rodent adipocyte preparations

The same procedure as above was applied for rat and mouse adipocyte preparations. A total of 10 male Wistar rats were purchased at Charles River (L'Arbresle, France) and were sacrificed according to INSERM guidelines for adipocyte preparation as previously reported[18]. Rat adipocytes were used at 15.3 ± 1.0 mg lipids/400 μL for the preliminary tests. Adipocytes were isolated from intra-abdominal adipose tissues obtained from male and female C57BL/6 mice. A total of 12 mice were used as already described[19] for the preparation of adipocyte suspensions that averaged 13.3 ± 0.8 mg cell lipids/400 μL.

Lipolytic activity assays

Filtration of digested adipose tissue, fat cell separation

and incubation were performed in disposable plastic wares at 37 ℃, as described[17]. All the tested agents were added to 400 μL of fat cell suspension in KRBHA under the form of 4 μL of a dilution extemporaneously done to reach the final indicated concentration. The agents were incubated with the fat cells at 37 ℃ under constant, gentle, shaking during 90 min. Incubations were stopped by placing the incubation tubes on ice. As already documented, lipolytic activity was determined by using glycerol release as an index[20], since FFA release follows parallel variations in our experimental conditions[21]. Once the buoyant adipocytes were frosted, 150 μL of medium were removed for glycerol spectrophotometric measurement at 340 nm, after addition of 1.5 mL of chromogenic mixture (0.6 mmol/L NAD, 1.4 mmol/L ATP, 0.2 mol/L glycine, 1 mol/L hydrazine, with 15 unit/mL glycerol phosphate dehydrogenase, and 0.6 unit/mL glycerokinase, pH 9.8), as previously described[22].

Glucose transport assay and de novo lipogenic activity

An isotopic dilution of [^{3}H]-2-deoxyglucose (2-DG) was added at a final concentration of 0.1 mmol/L (approximately 1300000 dpm/vial) to 400 μL of cell suspension after 45 min preincubation with the tested agents. Human fat cells were incubated for additional 10 min and then stopped with 100 μL of 100 μmol/L cytochalasin B. Aliquotes (200 μL) of shaken cell suspension were immediately centrifuged in microtubes containing dinonyl phthalate of density 0.98 g/mL, which allowed to separate the adipocytes as previously described[23]. The radiolabelled hexose internalized in viable fat cells (upper part of the tubes) was then counted in scintillation vials. The extracellular 2-DG present was determined using adipocytes whose transport activity was previously blocked by cytochalasin B at time 0. It did not exceed 1% of the maximum 2-DG uptake in the presence of insulin and was subtracted from the assays.

De novo lipogenic activity was determined by quantifying the D-[3-^{3}H]-glucose incorporation into lipids in mouse fat cells, according to[21]. They were incubated at 37 ℃ for 120 min in the same incubation medium as above, only containing only 0.6 mmol/L of isotopic glucose dilution as source of carbohydrates. The same vials were used for incubation, lipid extraction in an organic mixture for liquid scintillation (InstaFluorPlus) and counting of the labelled neo-synthesized lipids, following a procedure adapted from Moody *et al*[24].

Chemicals

Hexaquis(benzylammonium) decavanadate (B6V10) was synthesized and purified by Fernando Albericio and coworkers as previously detailed[16] and kindly given by Genmedica (Barcelona, Spain). Benzylamine, sodium orthovanadate, (-)-isoprenaline hydrochloride (isoproterenol), atrial natriuretic peptide (ANP), collagenase type Ⅱ and other reagents were from Sigma-Aldrich (Saint Quentin Fallavier, France). 2-DG and D-3-[^{3}H]-glucose were from Perkin Elmer (Boston, MA, United States).

Statistical analysis

Results are presented as means ± standard error of the means (SEM) of (n) observations. Statistical analysis for comparisons between B6V10 and respective control used Student's *t* test.

RESULTS

Effects of B6V10 in rat adipocytes

It is necessary to moderately activate lipolysis to detect whether a putative antilipolytic agent is able to limit triglyceride breakdown. This approach was first performed in rat adipocytes. The β-adrenergic agonist isoprenaline increased lipolytic activity in a typical concentration-dependent manner, and reached maximal activation at 1-10 μmmol/L. Addition of 100 μmmol/L of hexaquis(benzylammonium) decavanadate (B6V10) to increasing doses of isoprenaline impaired the β-adrenergic stimulation, clearly shifting the dose-response curve (Figure 1A). Noteworthy, the conjugate B6V10 did not alter the maximal effect of the highest isoprenaline dose. Similarly, the lowest dose of isoprenaline did not activate lipolysis sufficiently to allow any detection of B6V10 effect. Then, increasing concentrations of B6V10 were tested against an intermediate dose of isoprenaline (10 nmmol/L). In this condition, B6V10 dose-dependently inhibited the lipolytic activation induced by the β-agonist (Figure 1B). The conjugate therefore exhibited a clear and rapid antilipolytic effect in rat adipocytes, a cell model in which B6V10 has been already reported to mimic another insulin action: Glucose transport activation[13].

Effects of B6V10 in mouse adipocytes

Further studies performed on mouse adipocytes confirmed that increasing doses of B6V10 did not affect basal lipolysis, which was readily activated by isoprenaline (Figure 2A). Such lack of effect indicated that the conjugate was not lipolytic. However, other recognized antilipolytic agents, including insulin, were also unable to lower basal glycerol release (not shown). Consistent with our data obtained using rat adipocytes, activation of lipolytic activity was required to unmask putative antilipolytic effects. Consequently, B6V10 was tested at 1 μmmol/L in the presence of increasing doses of isoprenaline (Figure 2B). B6V10 did not impair the maximal lipolysis promoted by 0.1 and 1 μmmol/L of the β-adrenergic agonist, but it impaired the submaximal stimulation by 1 nmol/L and 10 nmol/L isoprenaline. When tested separately, the components of B6V10, benzylamine and sodium orthovanadate, did not alter the lipolytic effect of 10 nmol/L isoprenaline, while their combination at 100 μmol/L each was as antilipolytic as B6V10 (Figure 2B).

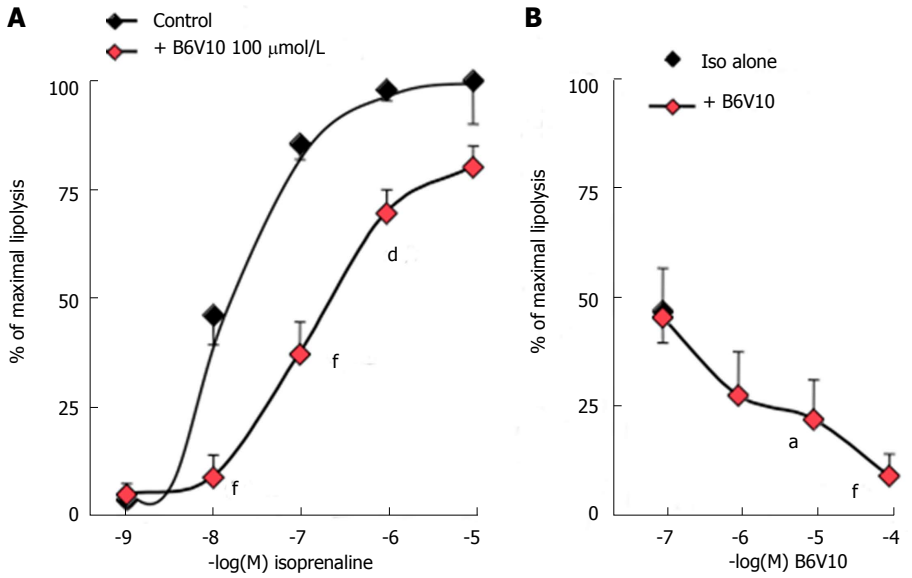

Figure 1 Influence of B6V10 on isoprenaline-induced lipolysis in rat adipocytes. Glycerol release was determined in rat fat cells incubated 90 min without (basal) and with increasing concentrations of isoprenaline alone (control, black symbols) or with the indicated doses of B6V10. Basal lipolysis (0.39 ± 0.06 μmol glycerol/100 mg lipid/90 min) was set at 0% while maximal lipolytic effect of 10 μmol/L isoprenaline (1.73 ± 0.14 μmol glycerol/100 mg lipid/90 min) was set at 100%. A: Antilipolytic effect of 100 μmol/L B6V10 (red diamonds) on dose-dependent activation by isoprenaline; B: Dose-dependent inhibition by B6V10 of the lipolysis induced by 10 nmol/L isoprenaline (iso alone). Mean ± SEM of 8-10 determinations. Significantly different from corresponding condition without B6V10 at: $^{a}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$.

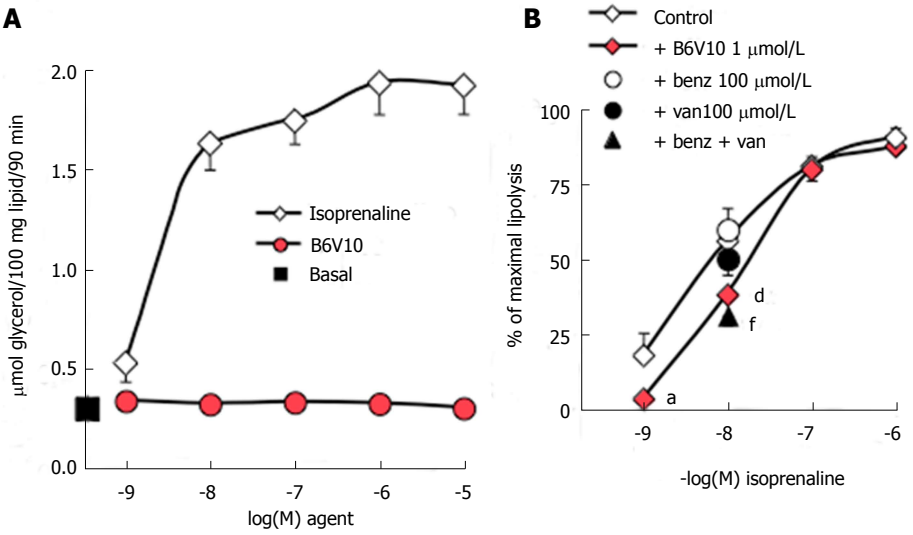

Figure 2 Influence of B6V10 on basal and isoprenaline-stimulated lipolysis in mouse adipocytes. Glycerol release was determined after 90-min incubation of mouse fat cells without (basal, closed square) or with the indicated concentrations of isoprenaline (open diamonds) or B6V10 (red symbols). A: Lack of lipolytic effect of increasing doses of B6V10 (red circles); B: Comparison of the inhibition of isoprenaline-stimulated lipolysis by 1 μmol/L B6V10 (red diamonds), 0.1 mmol/L benzylamine (open circle), 0.1 mmol/L vanadate (closed circle), or their combination (closed triangle). Mean ± SEM of 8 determinations. Significantly different from corresponding control at: $^{a}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$.

Thus, the antilipolytic action of B6V10 was detectable only when lipolysis was mildly activated by the β-adrenergic agonist isoprenaline. This could suggest that B6V10 was acting by antagonizing activation of β-adrenergic receptors. To ascertain that an antagonism at β-adrenergic receptors was not mandatory to observe a response to the conjugate, we verified its direct effect on glucose utilization in mouse fat cells. When tested alone at 10 μmol/L, B6V10 reproduced 49.0% ± 7.8% of the *de novo* lipogenic action of 100 nmol/L insulin, which was equivalent to a threefold increase over the basal values of the incorporation of radiolabelled glucose into the lipids of mouse adipocytes (n = 5, not shown). At 100 μmol/L, B6V10 reached 85.0% ± 3.5% of the maximal lipogenic effect of insulin. These data supported that the conjugate was active *per se* on adipocytes through a mechanism distinct from antagonism at β-adrenoceptors, since these G-coupled receptors were not activated during the test of lipogenic activity. Moreover, this verification

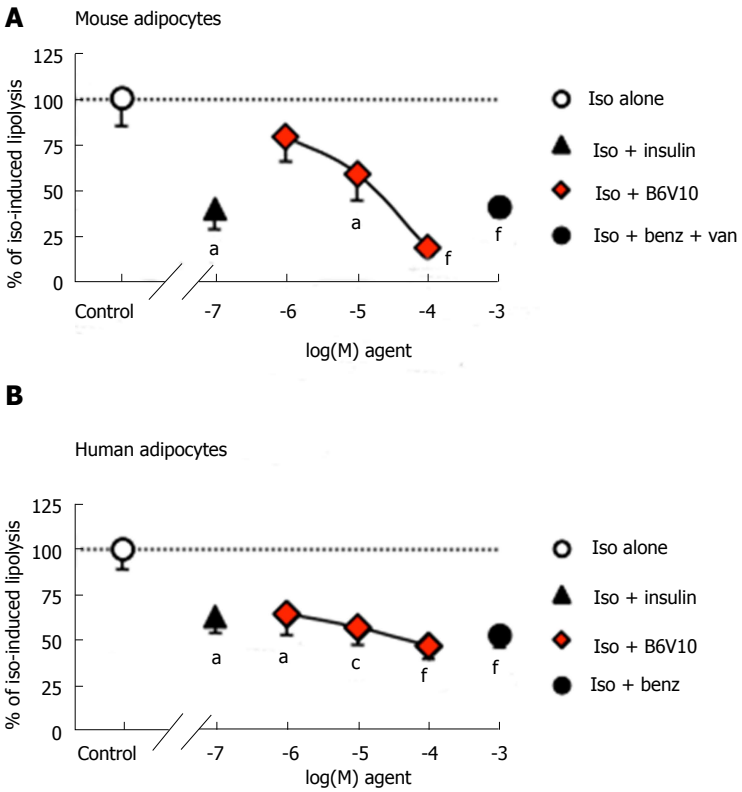

Figure 3 Comparison of the antilipolytic effects of B6V10 and insulin in mouse and human adipocytes. Lipolysis was activated by 10 nmol/L isoprenaline and considered as a control set at 100% (iso alone, dotted line) while basal was set at 0% in: Mouse adipocytes (A) or human adipocytes (B). The observed lipolytic response to the β-adrenergic agonist was significantly reduced in the presence of insulin (100 nmol/L, closed triangle), benzylamine (1 mmol/L alone for humans, or combined with 0.1 mmol/L vanadate for mouse adipocytes, closed circle), or increasing doses of B6V10 (1-100 μmol/L, red diamonds), at: $^{a}P < 0.05$, $^{c}P < 0.02$, $^{f}P < 0.01$. Mean ± SEM of 8 murine preparations (A) or 6-7 individual cases (B).

confirmed our previous characterization of B6V10 as an insulin-mimicking agent, acting through a hydrogen peroxide-dependent mechanism on the stimulation of glucose transport into fat cells[13]. In this context, the antilipolytic effect and the lipogenic effects of B6V10 could be considered as additional facets to the multiple B6V10 insulin-mimicking properties.

Translational studies on B6V10 antilipolytic action

Antilipolytic effects of insulin and B6V10 were then compared in mouse fat cells and in human adipocytes. In mouse, lipolysis was moderately activated by 10 nmol/L isoprenaline, reaching 1.28 ± 0.11 μmoles glycerol released/100 mg cell lipids/90 min. This sub-maximal stimulation of lipolysis represented an optimal condition to detect any putative induction or blockade by the tested agents. Figure 3 shows that the antilipolytic effect of a relatively high dose of insulin (100 nmol/L) was significant although incomplete. A similar partial antilipolytic effect was observed with 1 mmol/L benzylamine only in the presence of 0.1 mmol/L vanadate. The dose-dependent antilipolytic effect of B6V10 led to a stronger lipolysis inhibition than with insulin or benzylamine, at least when the conjugate was tested at 100 μmol/L (Figure 3A).

In freshly prepared human adipocyte suspensions, basal lipolysis was maximally activated by 10 μmol/L of isoprenaline (532% ± 107% of basal) but was unaltered by insulin alone (89% ± 16% of basal, n = 10, not shown). The stimulation of glycerol release by the dose of isoprenaline used in mice (10 nmol/L) was also submaximal. This dose triggered the production of 0.67 ± 0.08 μmol of glycerol/100 mg of cell lipids/90 min in human adipocyte preparations, while basal release was 0.20 ± 0.07 μmol glycerol/100 mg lipids/90 min. This lipolytic activation was considered as a 100% reference for testing the influence of insulin (100 nmol/L), benzylamine (1 mmol/L), or increasing doses of B6V10 (1-100 μmol/L) (Figure 3B). All these agents partially but significantly limited the β-adrenergic-induced lipolysis. When tested at 1 mmol/L, antilipolytic activity of benzylamine was as efficient as 100 μmol/L B6V10. The addition of vanadium did not enhance its effect (not shown).

B6V10 reduces submaximal but not basal and maximally-stimulated lipolysis in human adipocytes

Further analyses of the B6V10 antilipolytic effect were performed on human adipocytes and showed that 1 μmol/L of the conjugate could not impair the maximal lipolysis stimulation by 0.1, 1 or 10 μmol/L isoprenaline, while it impaired the submaximal β-adrenergic activation of glycerol release (Figure 4A). Similarly, no significant inhibition by B6V10 was detected on 1 nmol/L isoprenaline, when glycerol release values were close to basal levels. Moreover, B6V10 tended to limit the maximal effect of another strong lipolytic stimulator: The ANP, only active in human adipocytes[25,26] (Figure 4B).

In agreement with our data obtained in rodent adipocytes, micromolar doses of B6V10 were only limiting moderate lipolysis activation in human adipocytes. Thus, B6V10 appears to essentially hamper modest lipolytic activations, as those corresponding to the physiological modulation of triglyceride breakdown during interprandial cycles of energy supply and energy demand.

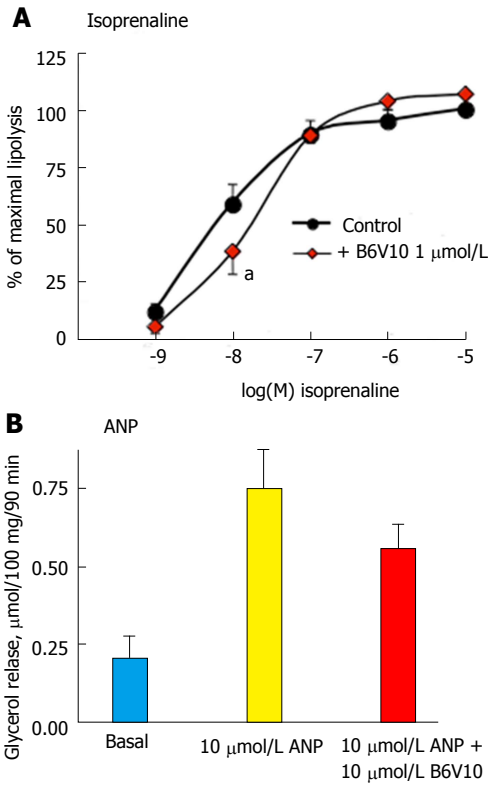

Figure 4 Antilipolytic action of B6V10 in human adipocytes depends on prior lipolytic activation. Lipolysis was activated by: Increasing doses of isoprenaline (A) or high dose of atrial natriuretic peptide (ANP) (B), and without or with the indicated doses of B6V10 (red symbols). Mean ± SEM of 6 determinations. Significantly different from corresponding condition without B6V10 at: $^{a}P < 0.05$.

Stimulation of glucose transport into human adipocytes by B6V10

Lastly, we explored whether the conjugate B6V10 could activate glucose transport in human adipocytes alongside its repression of triglyceride breakdown. In fact, previous studies that have demonstrated an insulin-like action of B6V10 on hexose uptake were restricted to murine adipocytes[13].

Here we show that freshly isolated human adipocyte preparations were highly sensitive to insulin, since 100 nmol/L of the hormone induced a four-fold increase in basal 2-deoxyglucose uptake (Figure 5). There was no significant effect of B6V10 on glucose uptake when added at inframicromolar doses. However, at 10 and 100 μmol/L, B6V10 reproduced approximately one third of the insulin stimulation of glucose transport, resulting in a highly significant activation. Sodium orthovanadate did not stimulate glucose uptake at 100 μmol/L and had no synergic effect with 10 or 100 μmol/L benzylamine. Indeed, when present at 0.1 mmol/L, benzylamine was as effective as 10 μmol/L B6V10 at stimulating hexose transport (Figure 5).

DISCUSSION

The property of B6V10, a conjugate of benzylamine and decavanadate, to lower blood glucose has been reported

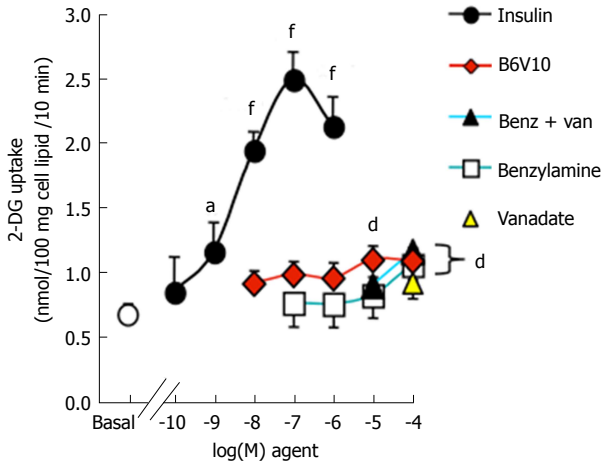

Figure 5 Activation of glucose transport in human adipocytes by supra-micromolar doses of B6V10. Suspensions of human fat cells (17 mg/400 μL) were incubated 45 min without (basal, open circle) or the indicated concentrations of insulin (closed circles), B6V10 (red diamonds), benzylamine (open squares), and 100 μmol/L vanadate alone (yellow triangle) or in combination with benzylamine (closed triangles), then [3H]-2-deoxyglucose uptake was assayed on 10-min period. Mean ± SEM of 10 adipocyte preparations. Significantly different from basal uptake at: $^{a}P < 0.05$, $^{d}P < 0.01$, $^{f}P < 0.001$.

together with *in vitro* demonstration of its ability to activate glucose transport and insulin signalling in adipocytes[13,14], while its ability to lower circulating FFAs in diabetic rats remains unexplained. Here we report for the first time that B6V10 is directly antilipolytic in murine and human adipocytes, a major finding regarding the growing interest for antilipolytic agents that may limit the harmful outcomes of lipotoxicity in diabesity[11,27,28]. Our observation using 1 μmol/L of the conjugate in human adipocytes, adds therefore a new insight to the development of vanadium-containing antidiabetic compounds, although it remains to avoid an overlap between their therapeutic and toxic doses. Accordingly, we are discussing below about the interest of inhibiting adipocyte lipolysis to reduce lipotoxicity, and about the possibility to develop vanadium-containing antidiabetic and anti-obesity agents that could become "drugable", two issues independently covered in very recent reviews[5,29].

In diabesity, when AT has developed an insulin resistance, the fatty acid storage in hypertrophied adipocytes under the form of triglycerides becomes limited due to a decrease in lipogenic and antilipolytic action of insulin. Such reduced insulin responsiveness derepresses lipolysis in adipocytes and leads to ectopic FFA deposition (in liver, vessels, muscles, endocrine glands), which in turn hampers glucose utilization and lipid oxidation in all these organs. To combat this lipotoxicity, there is a need to control excessive FFA mobilization from AT that requires the characterization of potent antilipolytic factors and constitutes a novel therapeutic approach for the treatment of obesity complications. In fact, there is mounting evidence that antilipolytic agents limiting the release of non-esterified fatty acid and glycerol into the blood stream, should be

considered as antidiabetic or anti-obesity agents[5]. In this regard, the reversal of lipotoxicity is proposed to contribute to the beneficial effects of old drugs, such as pioglitazone. These "novel" properties are added to the anti-inflammatory properties of pioglitazone that improve metabolic and secretory functions in adipocytes and β-cells[27], and lead to re-examine this old antihyperglycemic agent as a treatment for non-alcoholic fatty liver disease (NAFLD) that often complicates type 2 diabetes[28]. It is important to note that lipotoxicity can lead to severe NAFLD even when insulin resistance in AT is not concomitant with obesity. Indeed, the transgenic mice carrying fat-specific knockout of the insulin receptor are characterized by severe atrophy of fat depots, pronounced diabetes, and marked fatty liver disease[30]. Thus, it seems safer to limit ectopic lipid deposition by restricting excessive FFA release and blunting insulin resistance in adipocytes, even at the expense of maintaining adipose mass, than to overstimulate fat store mobilization. One has to keep in mind that one of the most powerful lipolytic agents, TNF-α, does not help in mitigating the deleterious outcomes of insulin resistance: On the opposite, it strongly desensitizes to insulin action and promotes inflammation.

Since we previously reported that chronic treatment with B6V10 lowered plasma FFA in diabetic rats[13], we asked whether this agent could be effective in lowering adipocyte lipolysis. In this comparative work, we brought compelling evidence that the conjugate inhibits lipolysis in rodent adipocytes with an efficiency greater than the ones obtained with its components used separately (benzylamine and decavanadate). Indeed, we have previously characterized B6V10 as an agent that exerts in adipocytes potent insulin-mimetic effects downstream the insulin receptor, in a manner that is sensitive to SSAO/VAP-1 inhibition and which reproduces the synergism between benzylamine and vanadate[13,14]. The effective doses of B6V10 in inhibiting lipolysis in rodent adipocytes are superimposable to those necessary for glucose transport stimulation. Moreover, our *de novo* lipogenesis experiments add to the list of B6V10 insulin-like effects[14] its capacity to activate glucose incorporation into the neosynthesized lipids in mouse adipocytes.

The fact that B6V10 was unable to impair maximal activation of lipolysis in all the models studied is not a concern since the amplitude of increased lipolytic activity of adipocytes is much lower in pathological states of insulin resistance than the activation that physiologically emerges during prolonged fasting or cold exposure[31]. Noteworthy, human adipocytes exhibited both lipolysis inhibition and glucose uptake activation in response to B6V10. Our data clearly show that the *in vitro* antilipolytic effect of a relatively high dose of insulin was not complete in subcutaneous adipocytes of sedentary women. Since insulin inhibited only by one-third of the response to isoprenaline, and since this effect was fully reproduced by 1 μmol/L of B6V10 (*i.e.,* at a dose only tenfold higher than that necessary for insulin), the conjugate can be definitely considered as a good insulin mimicker. Increasing the dose of B6V10 up to 100 μmol/L resulted in a higher but partial inhibition of lipolysis. Therefore, B6V10 could surpass insulin-like antilipolytic action in adipocytes from overweight subjects, who exhibited weak insulin sensitivity, although being non-obese and non-diabetic. Yet, the insulin antilipolytic response appeared to be more altered in these individuals than the insulin activation of glucose transport. The latter reached a four-fold stimulation of basal uptake in our conditions, which does not denote a fully developed insulin-resistant state for human fat cells[32]. Describing the exact onset of these defects was not in the scope of our studies, but deserves to be performed in future clinical studies, taking into account the influence of gender and fat depot anatomical location. Actually, it can be noticed that the maximal antilipolytic response to B6V10 was lower in human adipocytes than in rat and mouse models.

B6V10 is one of the promising antihyperglycaemic agents belonging to the wide family of vanadium derivatives. It can be summarized that, once ingested, vanadium is found in the organism under a cationic (vanadyl) or anionic (vanadate) form, the latter resembling to a phosphate group. In fact, orthovanadate ($H_2VO_4^-$) interacts with a pleiad of cellular components interacting with $H_2PO_4^-$, *e.g.,* enzymes influenced by (de)phosphorylation state. Yet, the ability of vanadium to mimic insulin actions in rat adipocytes has been reported in the 80s and univocally confirmed in all the insulin-sensitive tissues expressing GLUT4. Furthermore, we observed that vanadate and vanadyl were equally efficient in totally inhibiting rat adipocyte lipolysis at 1 mmol/L[33]. The current issue of vanadium pharmacology is to take advantage of these insulin-like properties without the concerns raised by the high degree of vanadium toxicity (due to accumulation in tissues like the kidneys and bones); in other terms: Lowering the risk/benefit ratio[29]. Among the various improvements raised by studies of chemico-biological interactions of vanadium derivatives[34], the vanadium peroxides, or pervanadates, formed by mixing vanadium and H_2O_2[16], have shown an increase in the potency for insulinomimetic actions in adipocytes[29]. With pervanadates, the effective doses were lowered from millimolar to micromolar range, as they are irreversible inhibitors of various phosphatases and act on target cells at much lower doses than vanadate. Recently, we synthesized and characterized salts composed by arylalkylamines combined with decavanadate that permitted to lower the effective antidiabetic dose of vanadium to non-toxic levels. The more active compound of this series, namely B6V10, mixes decavanadate, a complex form that increases adipocyte glucose uptake more potently than other vanadium forms[35], with benzylamine, also behaving as an insulin mimicker in human fat cells[36]. These two halves were already described to act synergistically, especially in fat cells where benzylamine is oxidized by the highly expressed SSAO/VAP-1,

thereby generating hydrogen peroxide[37], which in turn reacts with vanadate to generate peroxovanadate. This compound then inhibits protein tyrosine phosphatases and triggers glucose carrier translocation and hexose transport activation[38]. This cascade of events results in a substantial antihyperglycaemic action in diabetic rodents that is more potent than the separate effects of benzylamine and vanadate[39]. All these insulin-like actions disappear when SSAO/VAP-1 is pharmacologically inhibited or genetically invalidated[40]. Thus, when B6V10 undergoes oxidation by SSAO/VAP-1, it generates peroxovanadate and acts *in vitro*[16] as well as *in vivo*[14] to trigger antidiabetic actions. By releasing the real active vanadium-based ligands that interact with phosphatases near the target cells, the B6V10 is therefore a mean to improve decavanate "speciation" (see review from Scior and coworkers for further details[29]) and to circumvent the concerns raised by decavanadate toxicology[41].

Our *in vitro* analysis reveals a potent antilipolytic action of B6V10, which might be helpful in combating the lipotoxicity that participates to diabesity complications. Several concerns to this therapeutic potential could be raised since our experiments were performed only in adipocytes isolated from subcutaneous abdominal depots of overweight women.

The first concern could be the relevance of our observations for visceral adipocytes from massively obese subjects, considered as more harmful. Indeed, clinical studies have demonstrated that impaired triglyceride storage also occurs in the subcutaneous AT of insulin-resistant individuals when compared to their BMI-matched controls classified as insulin-sensitive[42]. Using deuterated water prolonged administration and functional exploration of subcutaneous AT, these studies elegantly indicated that, during the onset of type 2 diabetes in humans, there was a clear defect in insulin suppression of lipolysis and activation of *de novo* lipogenesis in the subcutaneous adipocytes themselves.

A second concern could be raised regarding the fact that we have only determined glycerol release as an index of lipolysis, while lipotoxicity is mainly supported by excessive FFA release. Previous studies on AT lipolysis and insulin sensitivity have evidenced a tight relationship between spontaneous glycerol production by human AT explants and insulin resistance in a large cohort of subjects presenting a wide range of BMI[43]. According to Girousse *et al*[43], both lipolysis end-products, glycerol and FFAs, were equivalent to show that partial inhibition of AT lipolysis improves insulin sensitivity[43].

Another limitation regarding the maximal antilipolytic capacity of B6V10 is that it is not complete and can be surpassed by various stronger antilipolytic agents (such as nicotinic acid, purinergic or α_2-adrenergic agonists, see[32]). However, these agents are unable to activate glucose uptake in human adipocytes (C. Carpéné unpublished observations) and do not offer the dual interest of B6V10 to lower both circulating glucose and lipids.

Lastly, insulin also plays lipogenic and antilipolytic actions when infused into the hypothalamus of rats[44]. Whether B6V10 also mimics insulin actions in the brain, in a manner that could influence its antidiabetic and lipid-lowering action during chronic treatment remains unknown and deserves further *in vivo* studies in insulin-resistant models.

Though being clearly antilipolytic in human adipocytes, 1 μmol/L B6V10 was not more effective than 1 mmol/L benzylamine, and the combination of vanadate with benzylamine did not lead to the synergism found in rat adipocytes. Indeed, in human AT, the maximal antilipolytic effect of B6V10 was comparable to that of benzylamine, already described to hamper about one-half of stimulated lipolysis[36]. Regarding the glucose uptake in human adipocytes, B6V10 is clearly stimulating, but there is no synergism between its components, benzylamine and vanadate, each one reproducing at 100 μmol/L the effect of 10 μmol/L conjugate. This is in apparent agreement with the proposed lack of glucose transport activation by decavanadate in human adipocytes[35], and confirms the absence of potentiation between SSAO/VAP-1 substrates and vanadium regarding glucose transport in human adipocytes[33]. Therefore, while noticeable synergism between SSAO/VAP-1 substrates and decavanadate occurs when using B6V10 in murine adipocytes, this apparently does not work as well in human fat cells, for a reason that remains to be elucidated.

Consequently, our comparative approach indicates that B6V10 cannot be immediately considered for clinical application as an efficient mean to increase the benefit/risk ratio of vanadium regarding its therapeutic antidiabetic indication. Nevertheless, it must be noted that, although B6V10 is not the most potent and powerful antilipolytic agents described so far in human adipocytes, it combines two insulin-like actions: Limiting lipolysis and increasing glucose uptake. In this regard, it should be considered as a valuable candidate to further develop an approach based on the mitigation of lipotoxicity in diabesity. This adds an alternative to classical antilipolytic agents proposed to limit lipotoxicity, such as nicotinic acid (Acipimox)[12], or lipase inhibitors[5]. Another consequence of our depicted interspecies differences is that the exploration of the antilipolytic properties in human adipose cells deserves to be applied to other vanadium conjugates recently tested with success on diabetic rodents, such as those combining metformin and decavanadate[45].

In conclusion, the conjugate of benzylamine and decavanadate B6V10 exerts insulin-like actions in human adipocytes, including lipolysis inhibition and glucose transport activation.

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COMMENTS

Background

Insulin resistance of adipocytes in hypertrophied fat depots leads to an increased lipolytic activity releasing in the circulation excessive amounts of free fatty acids (FFA) that accumulates under the form of triglyceride-rich ectopic lipid droplets in liver and muscles. The conjugate salt hexaquis(benzylammonium) decavanadate has been reported to lower circulating glucose and FFA in diabetic rodents, but its direct action of adipocyte lipolytic activity has never been assessed.

Research frontiers

This *in vitro* approach definitely brings evidence that B6V10 reproduces the rapid antilipolytic action of insulin in murine and human fat cells. At 100 μmol/L, B6V10 even surpasses the maximal inhibition of lipolysis induced by the pancreatic hormone. Since the molecule also stimulates glucose uptake in human adipocytes and has been demonstrated to exert antihyperglycemic actions in murine models of diabetes, it can be qualified as insulin mimicker.

Innovations and breakthroughs

In vitro, B6V10 exerts various insulin-like actions in human adipocytes including lipolysis inhibition and glucose uptake activation. This conjugate salt of benzylamine and decavanadate has the potential to alleviate the deleterious complications linked to the insulin resistance of adipocyte antilipolytic/lipogenic activities emerging in morbid obese and diabetic patients, and could be considered as a potential antidiabetic agent.

Applications

B6V10 could be useful as an auxiliary therapy in limiting the lipotoxicity related to obesity and insulin resistance. Its chronic administration might delay ectopic fat deposition and should reduce hepatic steatosis whether active in obese patients at doses acting in fat stores without exerting adverse effects elsewhere in the organism.

Terminology

ANP: Atrial natriuretic peptide; AT: Adipose tissue; BMI: Body mass index; B6V10: Hexaquis(benzylammonium) decavanadate; FFA: Free fatty acids; GLUT4: Insulin-sensitive glucose transporter; H_2O_2: Hydrogen peroxide; SEM: Standard error of the mean; SSAO/VAP-1: Semicarbazide-sensitive amine oxidase, identical to VAP-1 (vascular adhesion protein-1).

Peer-review

Manuscript presents solid data and is of good quality.

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ORIGINAL ARTICLE

Observational Study

Effects of intermittent fasting on health markers in those with type 2 diabetes: A pilot study

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Abstract

AIM

To determine the short-term biochemical effects and clinical tolerability of intermittent fasting (IF) in adults with type 2 diabetes mellitus (T2DM).

METHODS

We describe a three-phase observational study (baseline 2 wk, intervention 2 wk, follow-up 2 wk) designed to determine the clinical, biochemical, and tolerability of IF in community-dwelling volunteer adults with T2DM. Biochemical, anthropometric, and physical activity measurements (using the Yale Physical Activity Survey) were taken at the end of each phase. Participants reported morning, afternoon and evening self-monitored blood glucose (SMBG) and fasting duration on a daily basis throughout all study stages, in addition to completing a remote food photography diary three times within each study phase. Fasting blood samples were collected on the final days of each study phase.

RESULTS

At baseline, the ten participants had a confirmed diagnosis of T2DM and were all taking metformin, and on average were obese [mean body mass index (BMI) 36.90 kg/m^2]. We report here that a short-term period

of IF in a small group of individuals with T2DM led to significant group decreases in weight (-1.395 kg, P = 0.009), BMI (-0.517, P = 0.013), and at-target morning glucose (SMBG). Although not a study requirement, all participants preferentially chose eating hours starting in the midafternoon. There was a significant increase (P < 0.001) in daily hours fasted in the IF phase (+5.22 h), although few attained the 18-20 h fasting goal (mean 16.82 ± 1.18). The increased fasting duration improved at-goal (< 7.0 mmol/L) morning SMBG to 34.1%, from a baseline of 13.8%. Ordinal Logistic Regression models revealed a positive relationship between the increase in hours fasted and fasting glucose reaching target values (χ^2 likelihood ratio = 8.36, P = 0.004) but not for afternoon or evening SMBG (all P > 0.1). Postprandial SMBGs were also improved during the IF phase, with 60.5% readings below 9.05 mmol/L, compared to 52.6% at baseline, and with less glucose variation. Neither insulin resistance (HOMA-IR), nor inflammatory markers (C-reactive protein) normalized during the IF phase. IF led to an overall spontaneous decrease in caloric intake as measured by food photography (Remote Food Photography Method). The data demonstrated discernable trends during IF for lower energy, carbohydrate, and fat intake when compared to baseline. Physical activity, collected by a standardized measurement tool (Yale Physical Activity Survey), increased during the intervention phase and subsequently decreased in the follow-up phase. IF was well tolerated in the majority of individuals with 6/10 participants stating they would continue with the IF regimen after the completion of the study, in a full or modified capacity (*i.e.*, every other day or reduced fasting hours).

CONCLUSION

The results from this pilot study indicate that short-term daily IF may be a safe, tolerable, dietary intervention in T2DM patients that may improve key outcomes including body weight, fasting glucose and postprandial variability. These findings should be viewed as exploratory, and a larger, longer study is necessary to corroborate these findings.

Key words: Intermittent fasting; Type 2 diabetes; Remote food photography; Self-monitored blood glucose; Homeostasis model of assessment for insulin resistence index

Core tip: Intermittent fasting (IF) involves limiting food intake into a single 4 to 8 h period, daily. We observed the tolerability, safety and health benefits of IF in 10 type 2 diabetes mellitus (T2DM) patients during a 2-wk IF intervention. Outcomes were measured after the three study phases; baseline, intervention, and follow-up. Although short, the IF phase significantly improved weight loss and fasting glucose levels, was well tolerated, and hypoglycemia was not observed. During follow-up, glucose levels reverted. This simple, outpatient-directed dietary manipulation may prove valuable in T2DM individuals with exercise intolerance, who are resistant to complex diet regimes, or are not at glycemic goals.

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INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is reaching epidemic levels worldwide, and correlates with rising obesity rates and sedentary lifestyles. In fact, it is predicted that 439 million adults will have diabetes by 2030[1]. This is significant, as diabetes is closely associated with cardiovascular disease, retinopathy, neuropathy, and kidney disease. In turn, this places increasing stress on the health care system, and these patients utilize medical resources three to four times the amount of those without diabetes[2].

Modest weight loss and exercise regimes can both prevent the onset of T2DM and improve metabolic control[3]. According to most national diabetes associations and clinical practice, dietary interventions are considered essential in the treatment and prevention of diabetes-related complications[4]. There are many types of dietary interventions people may use; one of which is intermittent fasting (IF). This is a dietary intervention that time-restricts feeding to 4-6 h and extends the overnight fast from 12 towards 18 or 20 h, may be a beneficial additional dietary strategy used in T2DM management.

After diagnosis, most individuals (depending on individual circumstances) with T2DM are given management goals of an HbA1c below 7.0%, FPG 4.0-7.0 mmol/L, and post-prandial levels of 5.0-10.0 mmol/L[3,5,6]. Self-monitoring of blood glucose (SMBG) using glucometers can be critical for patient feedback and recognition of glucose control, as symptoms poorly predict glucose levels alone. Glucose goals are typically reached through an individualized treatment regime including lifestyle (diet and exercise) and medication use, yet a great number fail to reach, or maintain, these diabetic goals. As a result, a plethora of medication combinations are tried, as are weight loss approaches including consideration for bariatric surgery for morbidly obese individuals. Given the intense focus on weight and dietary measures, IF has the potential to be included in the armament in the fight to improve SMBG levels while contributing to weight loss in those in whom it would be beneficial.

In addition to glucose levels and body weight, there are other important aspects of T2DM worth considering. First is the degree of insulin resistance, which theore-

tically contributes to the difficulty of maintaining euglycemia[7]. Outside of the research clinic, insulin resistance can be measured in healthy patients using surrogate index measures derived from glucose and insulin measures in the fasting state. These surrogate index measures include glucose/insulin ratio, log fasting insulin, Homeostasis Model Assessment (HOMA-IR), log HOMA-IR, and Quantitative Insulin Sensitivity Check Index (QUICK1). A decrease in insulin resistance can improve glucose control, and exercise and weight loss both favorably decrease resistance states. Hence, we measured HOMA-IR, which has the most agreement with the clamp technique in assessing insulin resistance in T2DM patients[8], to help determine if IF may be beneficial.

Although the causal role is unknown, an association between chronic low-grade inflammation and diabetes has been shown to occur[9]. C-reactive protein (CRP) is a biomarker used to determine if inflammation is present. Elevated levels of CRP have been associated with insulin resistance, nephropathy progression and elevated fasting glucose in diabetes patients[10,11]. It is also known that CRP can be decreased with dietary therapy[12]. Hence, we measured CRP to see whether IF favorably decreased this inflammatory marker.

It can be challenging to measure dietary and exercise habits in clinical studies, and there is no defined intervention that is shown to be better than one another[13]. Monitoring devices worn daily are acceptable methods of tracking physical activity, with accelerometers being the current gold standard[14]. An alternative method to use in free-living adults is a questionnaire. Although not the gold standard, they have been shown to be a reliable method. The Yale Physical Activity Survey (YPAS) is a particular questionnaire that has shown to be reliable for capturing physical activity[14,15]. The YPAS has considerable test-retest reliability which makes it a useful tool for a repeated measures design in free living-adults[16,17]. Hence, the YPAS questionnaire was employed to track physical activity in this study.

The gold standard for measuring energy intake is the Doubly-Labelled Water method (DLW)[18]. However, this is difficult to use in those who are not experienced. One alternative to the DLW is the remote food photography method (RFPM). The RFPM has been shown to be an efficient and accurate method of capturing dietary intake in free-living adults[19,20]. Hence, the RFPM for 3 d in each of the study phases was utilized in this study to capture estimates of energy intake.

The Canadian Diabetes Association (CDA) publishes Clinical Practice Guidelines that currently recommends that individuals with T2DM follow the Canada Food Guide for nutritional needs[21]. Further, the CDA promotes various other nutritional strategies, such as portion control, carbohydrate counting, and grouping foods according to their glycemic index[4]. While these are all appropriate recommendations, some people have difficulty grasping these suggestions and fitting them into their diet[22]. Even an activity as simple as calorie-counting has been reported to lead to an increase in self-perceived psychological stress and cortisol levels, effects not seen when participants restricted calories unintentionally[23].

Another valuable resource for helping attain nutritional and diabetic goals is for patients to see a dietitian and/or attend a self-management program. However, many barriers exist for patients to access these programs or adhering to dietary advice[24]. Some of the barriers listed by people with diabetes range from feelings of helplessness and frustration, to not attaining desired glycemic control, to not being able to accommodate suggestions regarding food restrictions[22,25].

To overcome some of the barriers that some diabetes patients face with dietary interventions, alternative solutions should be explored. An example of an alternative dietary solution is IF. There are many variations of IF, but it is essentially restricting caloric intake to a specified period of time. One method of IF is to have people restrict their caloric intake for 18 to 20 h per day and eat *ad libidum* during those other 4 to 6 h. The feeding period will usually occur midday to early evening, and an increased protein intake may or may not be recommended to help increase satiety. During the fasting period, people are allowed to consume water, coffee, or tea. With this method of IF, caloric intake occurs when there is a diurnal peak in insulin sensitivity, and, similarly, a diurnal peal in cortisol levels during the fasting period. This may theoretically benefit glucose control. Similar protocols have been shown to have beneficial effects in non-diabetic populations with varying effects on glucose uptake, lipid levels, cortisol levels, and body fat[26-29]. Previous studies have shown that an IF intervention whereby all of one's daily calorie consumption are consumed in a four-hour window led to participants feeling too full and some weight loss, despite recommendations to consume more calories[26,27]. Hence, it is possible that IF can lead to a spontaneous caloric deficit in adult patients in their homes[30]. However, another study found that an IF intervention led to worsening of glycemic control[27], hence there are conflicting results as to its overall effects in a healthy individuals.

IF has received increasing attention for its potential therapeutic role in the treatment and prevention of cardiovascular disease and T2DM. However, we could only identify 2 studies in the literature evaluating the effects of IF in T2DM, although other trials are ongoing. The first evaluated a Mediterranean-style diet, with and without breakfast, on postprandial glucose, insulin, triglycerides and gastric inhibitory polypeptide in individuals with T2DM over a single day[31]. The Mediterranean lunch without breakfast revealed no increase in glucose, insulin, or triglycerides during the breakfast period (despite coffee intake), which did significantly increase above baseline during the comparator arms of low-fat and low-carbohydrate breakfasts. Another 12-wk had people undergo an IF fast whereby they only consumed caloric content in

the morning and early afternoon, and in this study, a decrease in FBG, HbA1c, and an improved response to an OGTT were observed[32]. Given these potential benefits, we designed a short-term observational pilot study to assess tolerability, safety, and specific anthropomorphic, biochemical and blood glucose health benefits of daily IF in patients with T2DM, and measured their persistence upon return to an unstructured diet.

MATERIALS AND METHODS

Recruitment

Participants were recruited from within the Saskatoon Health Region, which serves a population catchment of approximately 325000 people. Posters were delivered to general practitioners' offices in Saskatoon, as well as the 3 hospitals within the city of Saskatoon. Advertisements to the general public were placed in local newspapers and *via* internet outlets (*i.e*., Kijiji) alerting them of the study.

Individuals with a diagnosis of T2DM (confirmed by fasting glucose > 7.0 mmol, HbA1c > 6.5%, or OGTT > 11.0 mmol) and between the ages of 18-65 were eligible to enroll in this study. Certain medical conditions were excluded from enrollment, such as the presence of ischemic heart disease or heart failure, chronic inflammatory diseases, chronic infections, moderate to severe renal disease (GFR < 45), uncontrolled hypertension and hypoglycemic unawareness. Lastly, participants were excluded if they currently managed their diabetes with either insulin or glyburide due to their increased risk of hypoglycemia.

Study design

Interested participants that met inclusion criteria provided written consent and were educated on study procedures, and the risk, detection and management of hypoglycemia. The study was divided into 3 phases, baseline (run-in), intervention, and follow-up (Figure 1). During the baseline period, participants engaged in their normal dietary patterns (breakfast, lunch and dinner) for a period of 2 wk. During the intervention phase (weeks 3-4), participants were instructed to follow the IF meal timing pattern. This consisted of a fasting goal for a period of 18-20 h per day, with *ad libidum* zero-calorie coffee, tea, and water intake during fasting hours being permitted. During feeding time, participants were allowed to eat whatever they chose, but were encouraged to include at least 1/3 plate of protein to promote satiety (visual representations provided during training). The intervention phase was followed by a 2 wk follow-up phase with normal dietary patterns. There were no embedded criteria for weight loss, calorie restriction, or changes to exercise habits for the participants.

Assessment and evaluation

Self-reporting: Hours fasted, SMBG, caloric intake and exercise were all self-reported. Throughout all study phases, participants reported SMBG 3 times daily (fasting morning, random afternoon, and postprandial evening) with the use of a glucometer and logbook that was provided to them free of charge. In the same logbook, they also kept a diary of total consecutive hours fasted each day. In each of the three time periods (baseline, intervention, follow-up) participants completed a random 3-d food diary using the RFPM. During these days, participants received customized text message prompts by study staff to ensure compliance with RFPM. Participants responded to all text prompts to confirm that they had adhered to RFPM, as well as sent images of their food (before and after consumption to capture food waste). Physical activity/spontaneous energy expenditures were captured using the YPAS tool at the end of each of the study phases.

Biochemical and anthropometric measurements: Participants underwent fasted blood draws on the last day of the baseline phase, intervention phase, and follow-up phase. Fasting insulin, fasting plasma glucose (FPG) (with subsequent calculation for HOMA-IR) and CRP were measured. On each of these days, participants also underwent anthropometric measurements (height, weight, blood pressure, waist circumference). These measurements were all performed by the same individual.

Statistical analysis

All statistical procedures were performed on SPSS v. 22 and STATA v. 13. Data preparation was done using Excel 2011 and STATA v. 13. Significance was set at alpha = 0.05 (95%CI) for all tests. Repeat measures ANOVA were performed for measuring changes in anthropometric and biochemical changes.

The group means and standard deviations of days 1 through 42 (6 wk total study time) were calculated individually for three daily measurements: fasted morning (M), random afternoon (A), and postprandial evening (E) SMBG measurements. Linear and quadratic regression of group means and standard deviations were used to explore the relationship between study phase and SMBG.

OLR was used to explore the impact of relative daily fasting duration on SMBG. Cut-offs for OLR were created using standards for the diabetic fasting goal (< 7.0 mmol/L) and frank hyperglycemia (> 11.1 mmol/L), with an additional midpoint (9.05 mmol/L). The variable created for OLR was the hours fasted difference (HFD), the difference between actual hours fasted and the average hours fasted during the baseline phase (Table 1).

No category was created to represent hypoglycemic events, as none were recorded throughout the duration of the study.

RESULTS

Baseline participant characteristics

Summarized in Table 2 are the anthropomorphic and

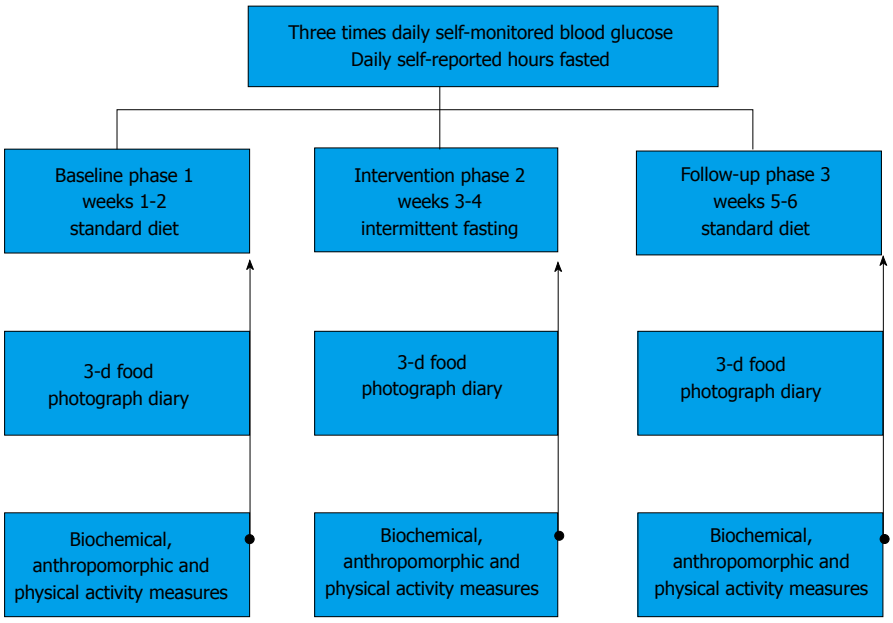

Figure 1 Study design. During the three-phase, 6 wk study, participants engaged in normal dietary patterns (breakfast, lunch and dinner) during weeks 1-2 (Phase 1, baseline) and 5-6 (Phase 3, follow-up). For weeks 3-4 (Phase 2, intervention) participants followed the IF meal timing pattern with a goal of daily fasts for 18-20 h per day, and a 4-6 h feeding period. *Ad libidum* zero-calorie intake, including coffee and tea during fasting hours, was permitted. Hours fasted and self-monitored blood glucose (fasted a.m., random afternoon pre-meal, postprandial evening) was reported daily throughout the study. On the last day (day 14, 28 and 42) of each phase, biochemical (fasting bloodwork), anthropomorphic (clinical assessment), and YPAS physical activity was collected. A 3-d consecutive photographic food diary was collected during each of the three phases. YPAS: Yale Physical Activity Survey; IF: Intermittent fasting.

Table 1 Cut-off points created to explore the impact of fasting on self-monitored blood glucose

SMBG value cut-off	Morning fasted	Evening postprandial
< 7.0 mmol/L	Normal/goal level	Normal/goal level
7.0-9.05 mmol/L	Above goal	Normal/goal level
9.05-11.1 mmol/L	Above goal	Above goal
> 11.1 mmol/L	Above goal	Above goal

SMBG: Self-monitored blood glucose.

Table 2 Baseline characteristics of participants

Measurement	Mean ± SD
Anthropomorphic	
Age (yr)	53.8 ± 9.11
Weight	100.6 ± 21.75 kg
BMI	36.9 ± 8.29 kg/m^2
Waist circumference	109.6 ± 11.1 cm
(reference < 88 female, < 102 male)	
Daily hours fasted	11.6 ± 1.9 h/d
Systolic BP (mmHg) T2DM goal < 130	130.00 ± 17.80
Diastolic BP (mmHg) T2DM goal < 80	80.50 ± 13.20
Biochemical	
C-reactive protein (mg/L)	4.31 ± 3.80
(reference < 1.0 mg/L)	
HOMA-IR calculated (normal < 2.5)	6.91 ± 3.00
Fasting glucose (normal < 7.0 mmol/L)	7.45 ± 1.52 mmol/L
Medications present during study period	
Metformin	10 (10)
Sulfonylureas	1 (10)
Other diabetic medications	1 (10)
Other non-diabetic medications	8 (10)

T2DM: Type 2 diabetes mellitus; HOMA-IR: Homeostasis Model Assessment; BMI: Body mass index; BP: Blood pressure.

biochemical measurements (with standard deviation) of the ten participants at study entry. Normal values for clinical and biochemical parameters are referenced. Subjects had a mean age of 53.8 years old (ranging 44-62) and BMI of 36.9 (range of 28-45 kg/m^2), the latter corresponding on average to Class II obesity, with very high disease risk compared to normal weight individuals. Insulin resistance was confirmed, with a mean HOMA-IR of 6.91, and baseline fasting blood glucose levels were above the goal of < 7.0 mmol/L (mean 7.45 mmol/L). A nonspecific biochemical marker of inflammation (CRP) was also elevated at baseline in this cohort. All participants took daily metformin as part of their diabetic management, eight as monotherapy. One participant was also taking gliclazide while another was also using liraglutide.

The clinical and biochemical changes resulting from a 2 wk dietary intervention of IF are shown in Table 3. The means of the measured biochemical and anthropomorphic outcomes of all subjects were calculated for each variable within the three phases of the study, with standard deviations shown: Phase 1 (baseline), Phase 2 (intervention) and Phase 3 (follow-up). Repeat measures ANOVA comparisons between study phases were done to reveal significant differences ($P < 0.05$). Clinical measures revealed that IF decreased mean body weight, BMI, blood pressure, and waist circumference as compared to baseline with significant changes only for body weight (-1.4 kg; $P = 0.009$) and BMI (-0.52; $P = 0.01$). The beneficial changes observed

Table 3 Differences between Study Phases for Biochemical and Anthropometric Parameters

Measurement	Mean ± SD Phase 1	Mean ± SD Phase 2	Mean ± SD Phase 3	Mean difference Phase 1 to 2	Mean difference Phase 2 to 3	Mean difference Phase 1 to 3
Clinical outcome						
Weight (kg)	100.6 ± 21.7	99.2 ± 21.3	99.5 ± 21.5	-1.4 (P = 0.009)	+0.28 (P = 1.0)	-1.12 (P = 0.08)
BMI (kg/m^2)	36.9 ± 8.3	36.4 ± 8.1	36.5 ± 8.1	-0.52 (P = 0.01)	+0.1 (P = 1.0)	-0.42 (P = 0.09)
Waist circumference (cm)	109.6 ± 11.1	107.8 ± 11.1	107.5 ± 10.9	-1.75 (P = 0.086)	-0.30 (P = 1.0)	-2.05 (P = 0.24)
Systolic BP (mmHg)	130.0 ± 17.8	127.0 ± 21.4	128.5 ± 14.3	-3 (P = 0.83)	+1.5 (P = 1.0)	-1.5 (P = 1.0)
Diastolic BP (mmHg)	80.5 ± 13.2	79.8 ± 15.7	81.7 ± 12.2	-0.72 (P = 1.0)	+1.9 (P = 0.76)	+1.2 (P = 1.0)
Daily hours fasted	11.6 ± 1.9	16.8 ± 1.2	11.5 ± 2.0	+5.2 (P < 0.005)	-5.3 (P < 0.005)	-0.09 (P = 1.0)
Biochemical outcome						
C-reactive protein (mg/L)	4.3 ± 3.8	4.0 ± 3.7	4.1 ± 3.5	-0.3 (P = 0.9)	+0.09 (P = 1.0)	-0.25 (P = 1.0)
HOMA-IR	6.9 ± 3.0	6.5 ± 2.4	6.6 ± 3.0	-0.46 (P = 1.0)	+0.11 (P = 1.0)	-0.35 (P = 1.0)

HOMA-IR: Homeostasis Model Assessment; BMI: Body mass index; BP: Blood pressure.

Table 4 Morning, afternoon and postprandial self-monitored blood glucose levels decreased during intermittent fasting

14 d averaged SMBG pooled	Mean ± SD Phase 1	Mean ± SD Phase 2	Mean ± SD Phase 3	% change from Phase 1 to 2	% change from Phase 2 to 3
μfasting SMBG	8.2 ± 1.3	7.7 ± 1.8	8.1 ± 1.4	-6.10%	+5.20%
μafternoon SMBG	7.5 ± 1.0	7.2 ± 1.2	7.0 ± 0.9	-4.00%	-2.80%
μpost prandial SMBG	8.7 ± 1.9	8.6 ± 1.9	8.8 ± 1.7	-1.10%	+2.30%

μ: Average; SMBG: Self-monitored blood glucose.

were not sustained once the IF was complete. After a return to normal diet (Phase 3, follow-up), there was an inflection back toward baseline values for all parameters except a further non-significant decrease in waist circumference (-0.3, P = 1.0). All participants increased their fasting time during the intervention phase. The daily hours fasting increased from a baseline of 11.6 to 16.8 h during the intervention phase (+5.2 h; P < 0.001), and essentially returned to baseline (11.5 h) after the follow-up period.

The averages of SMBG reported daily from home glucometers decreased during the intervention phase for fasting morning, afternoon and postprandial time points (Table 4). Pooled averages of SMBG taken three times daily throughout the study are presented by study phase, indicating the % change from baseline to IF (Phase 1 to 2), and IF to follow-up (Phase 2 to 3).

IF improves morning fasted glucose levels and decreases postprandial variability. To further investigate potential benefits of IF on glucose levels, daily (rather than grouped by study phase) SMBGs were plotted over the 42 d study (Figure 2A). To investigate the glucose variances from the mean for fasting morning glucose levels, the Kolmogorov-Smirnov (KS) test was used. Figure 2B indicates that the daily distribution of morning SMBG was greatest during the intervention as compared to baseline (P = 0.002), or follow-up (P = 0.003) phases. Lastly, a similar assessment of the daily variation from the mean for evening postprandial values (Figure 2C) demonstrates wide scatter throughout all phases, with decreases in glucose variability from baseline to intervention, as well as from intervention to follow-up phase. A significant difference was found for evening SMBG distributions (P = 0.044) between the intervention and follow-up phases only (all other phases P > 0.1). There were no statistically significant differences observed between any phases for the afternoon SMBG distributions (P > 0.1).

IF enhances the proportion of fasting SMBG at goal and decreases postprandial glucose excursions. Raw SMBG counts and percentages of SMBG residing within defined glucose categories were tabulated for each study phase. Table 5 reflects morning fasted and evening post-prandial SMBG. SMBG results were placed within four discrete glucose ranges including normal/target fasting (< 7 mmol/L), frank hyperglycemia (> 11.1 mmol/L), and an equal cut-off bridging the two (cut off at 9.05 mmol/L) to determine the cause of the increased variation noted for morning SMBG during IF. The distributed results for fasting SMBG readings Table 5 highlight that the incidence of at-goal SMBG < 7.0 mmol/L increased 2.5-fold during the IF intervention (13.8% baseline increased to 34.1% during IF). Also noted during IF was the overall decrease in fasting hyperglycemia (> 7.0 mmol/L) that was offset by a greater incidence of elevated fasting levels (> 11.1 mmol/L noted at 0.8% baseline, increasing to 7.1% during IF). The improvement of readings at target glucose levels was lost during follow up, upon return to normal eating patterns.

Considering the pooled incidence of evening post-prandial SMBG up to and including 9.05 mmol/L as "at-goal" for postprandial blood glucose, IF resulted in a higher proportion in the desirable range (Table 5). Specifically, 65.7% of SMBG were < 9.05 mmol/L during the IF phase of the study, compared to 52.6%

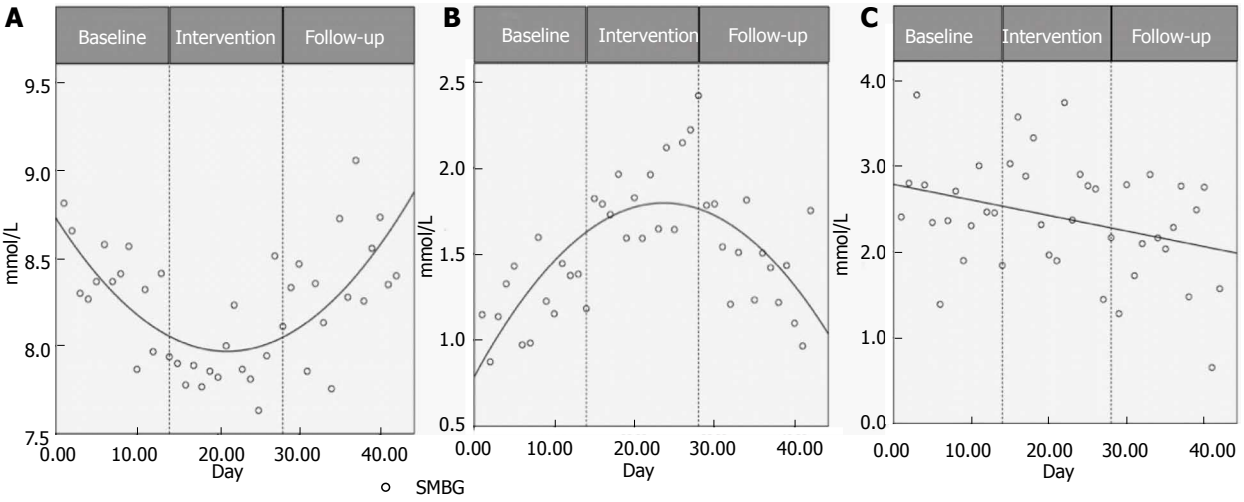

Figure 2 Intermittent Fasting improves morning fasted glucose levels and decreases postprandial variability. The daily means for fasting morning glucose levels (from personal glucometers) on days 1 through 42 are shown in Figure 2A, with the three phases indicated. Means were calculated from pooled SMBG data from the nine individuals that provided complete log sets. Figure 2B represents the daily variance from the mean for fasting morning SMBG, days 1-42, using the Kolmogorov-Smirnov (KS) test. Figure 2C shows the daily variance from the mean for evening postprandial SMBG values on days 1-42. Inflection points of quadratic equations were calculated using the formula [f 1 (y) of C + Ax + B × 2 = 0] rounded down to the nearest full integer. A: Mean morning fasted SMBG; B: Morning fasted SMBG variability; C: Evening random SMBG variability. SMBG: Self-monitored blood glucose.

Table 5 Intermittent fasting improves fasting and postprandial glucose levels

Measured SMBG (mmol/L)	Baseline	Intervention	Follow-up
Morning SMBG by phase			
< 7.0	13.8%	34.1%	15.1%
7.0-9.05	52.0%	40.7%	49.6%
9.05-11.1	33.3%	18.0%	32.8%
> 11.1	0.8%	7.1%	2.5%
Evening SMBG by phase			
< 7.0	24.5%	27.7%	12.9%
7.0-9.05	28.1%	32.9%	41.6%
9.05-11.1	27.4%	19.7%	28.7%
> 11.1	20.0%	19.7%	16.8%

SMBG: Self-monitored blood glucose.

Table 6 Ordinal Logistic Regression: Relationship between Hours Fasted Difference and morning, afternoon, and evening self-monitored blood glucose

SMBG	Overall model		
	Odds ratio	*P* value	95%CI
Morning	0.91	0.004	0.85-0.97
Afternoon	0.95	0.181	0.88-1.02
Evening	1.00	0.900	0.94-1.07

SMBG: Self-monitored blood glucose.

at baseline. Similar levels (54.1%) were found at goal during the follow-up phase, as at baseline. The proportion of SMBG > 11.1 mmol/L changed little with IF. The greatest change was found in the decrease in SMBG between 9.05-11.1 mmol/L, specific to the IF phase.

The increase from baseline, rather than the absolute number, of hours fasted improves the probability of optimal glucose control. HFD was calculated for baseline and intervention phases to explore the relationship between the increase in hours fasted from baseline and SMBG improvements. In order to determine the relationship between glucose levels and time spent fasting, OLR was performed on the non-equalized data sets from the 8 participants who had completed both their daily-hours-fasted and full SMBG logs. As presented in Figure 3, these results support the previous SMBG regression findings; mean morning SMBG readings decreased, and improvements in these readings were primarily a result of increased fasting hours from baseline. The HFD and OLR models showed a statistically significant association between change in HFD with decreased SMBG morning readings (χ^2 likelihood ratio = 8.36, P = 0.004) but not for afternoon or evening SMBG readings (P > 0.1, Table 6).

The IF phase was associated with spontaneous decreases in caloric intake and increases in physical activity. To calculate energy intake during the study, five (baseline and intervention) and four (follow-up) participants recorded their food intake *via* the RFPM. This was performed for 3 d of each study phase. From this, estimates of the total kcal/day, and the proportionate contribution from protein, carbohydrate and fat was determined as shown in Table 7. Total kcal/day decreased 18.6% with the IF intervention, and further decreased 6% into the follow-up phase. Carbohydrate and fat intake decreased 33% and 36% respectively during the IF period, whereas protein intake remained constant. To estimate physical activity during the study, all participants completed the YPAS once during each of the three phases. From this survey, an estimate of physical activity (kcal/wk) during the three study phases was determined. During the intervention phase, physical activity increased (+1856.3 kcal/wk), but then decreased at study end (in the follow-up phase; -2449.6 kcal/wk).

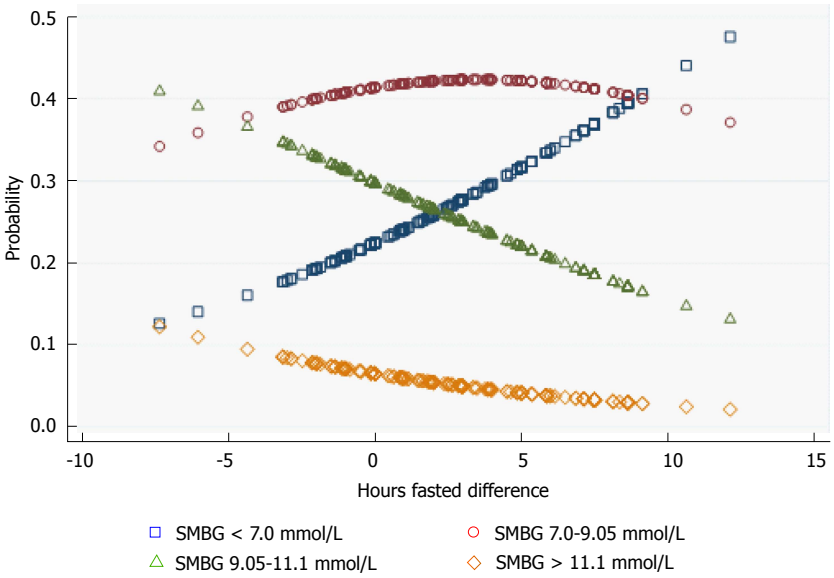

Figure 3 Positive relationship between hours fasted and morning glucose levels. Ordinal logistic regression (OLR) tested the association between the increase in hours fasted from baseline, and the morning fasting glucose levels within the four glucose ranges noted. OLR was performed using data from the 8 individuals who had completed both their full SMBG logs and daily hours fasted logs. OLR models used the non-equalized data. Hours Fasted Difference (HFD) was calculated for baseline and intervention phases only. SMBG: Self-monitored blood glucose.

Table 7 Patient reported diet composition and physical activity, by study phase

Measure	Baseline (mean ± SD)	Intervention (mean ± SD)	Follow-up (mean ± SD)
Extrapolated energy intake (kcal/d)	1904.3 ± 404.1	1605.7 ± 375.5	1510.5 ± 755.4[1]
Protein intake (g/d)	94.2 ± 26.6	93.2 ± 26.1	79.4 ± 30.7[1]
Carbohydrate intake (g/d)	190.6 ± 58.5	142.7 ± 62.1	164.2 ± 93.9[1]
Fat intake (g/d)	86.9 ± 16.6	63.6 ± 25.2	60.9 ± 35.5[1]
Physical activity (kcal/wk)	4922.3 ± 3774.4	6778.56 ± 4329.5	4329.0 ± 3440.8

[1]Data from 4 participants.

DISCUSSION

Key findings

Many individuals with T2DM would benefit from a simple and accessible nutrition intervention that is simple to implement and teach, and improves their glycemic control. Teaching diabetes patients about IF only required between 15-30 min of the study coordinator's time, hence it was easy to teach. Although short term (2 wk IF intervention), and without oversight (self-reported and self-controlled eating hours), the intervention resulted in significant improvements in diabetic glucose control. The IF phase yielded a significant increase in the incidence of fasting blood sugars at target (34.1% *vs* 13.4% baseline), and favorable decreases in postprandial hyperglycemia (39.4% *vs* 47.4% baseline). There was also a spontaneous 18% decrease in caloric intake and increase in energy expenditure (+1856 kcal/wk), coinciding with a significant decrease in weight ($P < 0.009$) and BMI ($P = 0.01$). A strong association between the increase in hours fasted from baseline, and the probability of attaining a normal fasting glucose level was found (+LR 8.36), despite few individuals reaching the 18-20 h fasting goal. We did not find statistically significant changes to blood pressure, insulin resistance or inflammatory markers after 2 wk of IF, although all trended towards normal during this phase. Importantly, the diet was found to be tolerable and safe, with zero incidences of hypoglycemia.

Upon a return to normal eating habits, the improvements to fasting glucose levels reversed to baseline, a trend also seen for the non-significant improvements in CRP, HOMA-IR and BP. The sample size of this study is too small to determine if the sustained decreases in waist circumference, postprandial glucose variability, and energy intake into the follow-up period are meaningful.

Comparison to other dietary outcomes

The most similar study to ours that we could identify in the literature evaluated IF was a 3 mo crossover study in T2DM patients[32]. These studies differed with respect to when participants were instructed to undergo the prolonged fasting period (participants in our study were allowed to self-select their fasting period, and chose late afternoon to early evening). Similar trends were observed with respect to weight loss and decreased waist circumference, whereas the study by Kahleova *et al*[32] also showed significant improvements in fasting insulin (and presumably insulin resistance), which our did not. In contrast, other studies in non-diabetic, normal-weight participants, have shown that IF resulted in increased insulin resistance, fasting glucose, and lipids, despite weight loss occurring[26,27]. These different observed effects between diabetic and non-diabetic individuals to similar dietary changes may be related to the differences in body weight and hyperglycemia

between the studies, or due to as yet unknown factors.

This work demonstrates that there is a correlation between hours fasted and improvements in fasted glucose levels, leading us to question if simply skipping breakfast (and thereby prolonging the fast) would improve T2DM control. A study by Thomas *et al*[33] tested the effects of skipping breakfast in obese women (without diabetes). They found that when regular breakfast eaters skipped breakfast, they had greater insulin and free fatty acid excursions in response to lunch as opposed to days when both breakfast and lunch were eaten[33]. In contrast, those who regularly skipped breakfast did not experience irregular responses at lunch, regardless of whether breakfast was eaten or not[33]. Clearly, extended fasting will not benefit all and has the potential to worsen measures of health if applied to patients with T2DM.

We also query if the observed benefits in this study arise from altering the normal eating rhythm, rather than inducing a net negative energy state (such as during fasting, or exercise). Although some evidence from animal studies suggest that biological clocks may be altered as a result of changes in feeding habits[34,35], evidence of the effects of feeding entrainment on glycemic excursions in humans are lacking.

Strengths and weaknesses of study

Our study involved ten individuals (9 female, one male) who present with the typical characteristics of the majority of the worlds' T2DM population; middle age, overweight, with insulin resistance and average fasting blood sugars above goal. There are several unique aspects to this study that provide useful information, not the least of which was the patient-controlled meal timing and content, all without caloric restriction. It is the first study designed with patients directly transitioning from their normal dietary habits, to an IF intervention, to a follow-up period to see if any of the effects were sustained. Also, the capturing of SMBG readings along with the actual total hours fasted allowed us to make inferences on this relationship, which has not been done before. Another strength is our use of the RFPM, which has been shown to have good validity[19,20]. Adherence with the study protocol, as reflected by SMBG and the recorded hours fasted, was high. Also, as a surrogate for tolerability of the IF intervention, 7/10 participants viewed the intervention as tolerable when asked, and 6/10 stating they would consider continuing a modified form of IF.

The greatest weakness was the lack of power from low recruitment, which was the limiting factor in determining clear effects of IF on markers of health in T2DM, or the detection of sustained effects during follow-up. An uneven distribution between females (n = 9) and males (n = 1) is also a limiting factor for generalizability. Also of importance to note was the failure to reach the goal of 18-20 h of fasting per day, as these longer fasting times may have accentuated health benefits more clearly. Ultimately, the 16.8 ± 1.2 h fasting during Phase 2 of the study is perhaps a true reflection of the feasibility of fasting times for this diet in free-living adults. Further a longer duration of the study phases would have allowed for comparative HbA1c measures to be done, a widely accepted marker of average changes to overall glucose control reflecting the previous three months. Lastly, it is possible that all of the SMBG testing that patients did may have in itself led to behavior changes that affected the overall recorded glucose levels. Any information learned from this observational pilot study can be used to inform a larger, longer, observational or interventional trial.

Future directions and developments

Our understanding of the association between feeding entrainment and diurnal rhythms is limited, and requires further study. Also, our study took blood draws from patients at specific time points, for study ease. Ideally, patients would have blood draws performed regular over a 24 h period so that fluctuations could be noted that may be due to diurnal variations, as the consumption of calories changes over the IF period. Since our participants only had blood draws performed in the morning, some biochemical changes that may have occurred during other time periods would have been missed. It would also be ideal for future studies to have a longer study period which would allow for significant weight loss to occur, and then see if any biochemical changes are sustained during an extended follow-up period. Of course, a larger sample size would allow for a more robust conclusion as to the association between SMBG and IF and would shed som light on the true effects on morning, afternoon and evening SMBG results.

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COMMENTS

Background

Intermittent fasting (IF) is an increasingly popularized dietary method amongst varying population groups. The reasons for its increasing popularity are multifactorial, but one of which is for weight loss. Although research has been performed evaluating an IF intervention in animals and healthy subjects, there is very little known about its effects, beneficial or otherwise, in those with type 2 diabetes.

Research frontiers

As the prevalence of diabetes continues to increase, new dietary measures are necessary to pursue, as one size does not fit all. It is possible that IF may be a good option for people with diabetes alongside other popularized diets such as the Mediterranean diet, the DASH diet, and the Newcastle diet. With type

2 diabetes affecting so many people worldwide, any new potential treatment options are welcome.

Innovations and breakthroughs

To our knowledge, there is only one other study examining IF in type 2 diabetes (Kahleova *et al*). The study varied in that patients were allowed to self-select their meal times and meal options. Further, the study showed that when practiced with self-selected meal times and meal options, there is a spontaneous reduction in caloric intake (whereas the study by Kahleova provided meals and all patients were assigned to a caloric deficit). Further, the participants chose to fast mostly in the mornings and leave their meals to nighttime, whereas the study by Kahleova had their participants eat only in the morning and afternoon, and fasted in the evenings). All told, there is very little literature examining IF in diabetes patients, and so all information can be seen as important and innovative in their approach to the treatment of type 2 diabetes.

Applications

Managing the dietary aspect of diabetes can be very difficult and tiresome for some people. It can often create unnecessary stress and sense of failure in those who are unable or unwilling to adhere to dietary recommendations to help control their blood glucose and body weight. IF is a potential alternative for certain people who wish to consume their caloric intake for the day in a manner which is not considered "recommended" for people with diabetes. It also may lead to some weight loss through less caloric consumption, which is often welcome for many people with type 2 diabetes.

Terminology

IF: An eating pattern that time-restricts feeding to 4-6 h and extends the overnight fast from 12 h towards 18 or 20 h.

Peer-review

The study is valuable and of interest as intermittent fasting is a hot topic regarding weight loss and related benefits including treatment of diabetes.

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SYSTEMATIC REVIEWS

KMAP-O framework for care management research of patients with type 2 diabetes

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Abstract

AIM

To review impacts of interventions involving self-management education, health coaching, and motivational interviewing for type 2 diabetes.

METHODS

A thorough review of the scientific literature on diabetes care and management was executed by a research team.

RESULTS

This article summarizes important findings in regard to the validity of developing a comprehensive behavioral system as a framework for empirical investigation. The behavioral system framework consists of patients' knowledge (K), motivation (M), attitude (A), and practice (P) as predictor variables for diabetes care outcomes (O). Care management strategies or health education programs serve as the intervention variable that directly influences K, M, A, and P and then indirectly affects the variability in patient care outcomes of patients with type 2 diabetes.

CONCLUSION

This review contributes to the understanding of the KMAP-O framework and how it can guide the care management of patients with type 2 diabetes. It will allow the tailoring of interventions to be more effective through knowledge enhancement, increased motivation, attitudinal changes, and improved preventive practice to reduce the progression of type 2 diabetes and comorbidities. Furthermore, the use of health information technology for enhancing changes in KMAP and communications is advocated in health promotion and development.

Key words: KMAP-O framework; Type 2 diabetes; Behavioral intervention strategies; Causal mechanisms

Core tip: A complex set of behavioral and cognitive variables related to diabetes care may influence adherence and self-care practice of patients with type 2 diabetes. This systematic review is guided by a behavioral system framework. Care management strategies or health education programs serve as intervention variables that may directly influence a patient's knowledge, motivation and attitude, self-care practice, and outcomes. This review summarizes key findings in regard to the validity of developing a comprehensive behavioral system as a framework for future empirical investigation.

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INTRODUCTION

More than 29 million people in the United States have diabetes[1]. Type 2 diabetes, which accounts for 90% to 95% of all cases, occurs when the body develops insulin resistance and cells no longer transport glucose using insulin. This leads to an overproduction from the pancreas, and eventually the pancreas does not produce enough insulin when blood sugar levels increase[2]. In 2012, the total estimated cost of diagnosed diabetes in the United States was $245 billion, including $176 billion in direct medical costs and $69 billion in lost productivity[3]. Diabetes is associated with higher risk of blindness, kidney failure, heart disease, stroke, and amputations[1]. Diabetes control requires a systematic effort of adherence to medical regimens and preventive practice in diet and exercise. A comprehensive framework for promoting diabetes care is proposed in this review of the empirical literature.

MATERIALS AND METHODS

This review is centered on type 2 diabetes from a behavioral system perspective and guided by the scientific literature published in social, behavioral, and medical science journals. The research team collectively conducted the review of studies published in the scientific literature. Both conceptual and empirical developments in health education and research are highlighted in the review.

RESULTS

KMAP-O framework for type 2 diabetes

Behavioral and social scientists have considered a behavioral systems approach to medical adherence studies. Their research is centered in the identification of how knowledge, attitude, and preventive practice may influence the variability in health and behavioral outcomes. However, this approach fails to articulate causal-specific links among these major contributing factors to outcome variations. Furthermore, the lack of attention to motivational factors in search for the determinants of health care outcomes has compounded the investigative problem that has hindered the full explication of the important role of motivation in the KAP studies.

The KMAP-O framework can be used to guide care management of type 2 diabetes patients. The first construct of the KMAP-O model is knowledge. Knowledge is "the acquisition, retention, and use of information or skills"[4,5]. Type 2 diabetes patients should have the knowledge to understand the condition, its progression, and necessary self-care practices[5].

The second construct in the model is motivation. Motivation is an individual's desire or willingness to behave in a certain way. A person can be described as unmotivated if he or she feels no impetus or inspiration to act, while a person who is energized or activated toward an end is characterized as motivated[6].

Following knowledge and motivation, attitude is the next construct in the KMAP-O framework. Attitude is a "psychological tendency that is expressed by evaluating a particular entity with some degree of favor or disfavor"[7]. A patient's attitude toward diabetes involves any preconceived ideas about the condition and its management, any feelings and emotions toward aspects of diabetes and diabetes care, and the aptness to behave in particular ways about diabetes and its management[5].

Practice is the fourth construct in the model. Practice is a demonstration of "the acquisition of knowledge (increased understanding of a problem/condition) and any change in attitude caused by the removal of misconceptions about the condition"[5]. The following seven key behaviors to practice in diabetes management, as identified by the American Association of Diabetes Educators, are healthy eating, physical activity, blood glucose monitoring, medication taking, problem solving related to diabetes self-care, reducing risks of acute and chronic complication, and healthy coping[8].

The last construct in the framework is outcome. Outcomes that are commonly assessed in type 2 diabetes patients are psychosocial measures such as quality of life (QoL), and physical measures such as blood pressure, body mass index (BMI), body weight, hemoglobin A1c (HbA1c) levels, and lipid levels. The causal specifications among the KMAP-O components are portrayed in Figure 1. This model suggests that health education or behavioral intervention(s) may directly affect knowledge, motivation, attitude and practice. The changes in knowledge, motivation and attitude may also directly influence practice variations

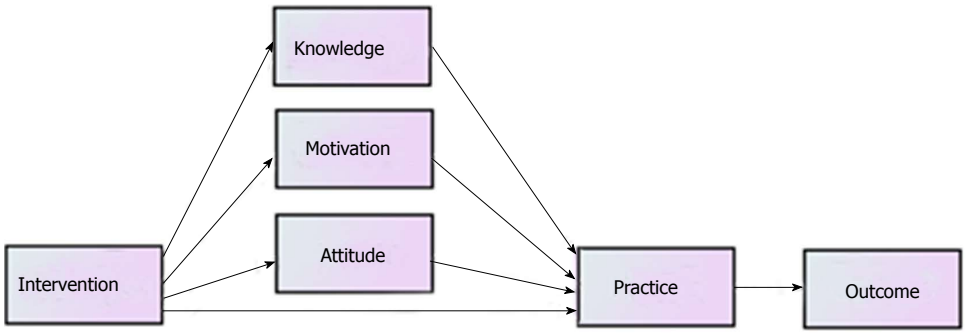

Figure 1 KMAP-O components.

in diabetes control. Consequently, better practice behavior in diabetes care management may result in a positive improvement in clinical and self-reported healthcare outcomes. These causal specifications enable researchers to generate multiple, testable hypotheses in empirical studies on care management effectiveness for type 2 diabetes.

The management of type 2 diabetes requires modification of complex behavior and practices to achieve optimal outcomes. Interventions that encourage these changes include self-management education, health coaching, and motivational interviewing. Self-management education is "a collaborative and ongoing process intended to facilitate the development of knowledge, skills, and abilities that are required for successful self-management of diabetes"[9,10]. Health coaching aims to help individuals achieve goals through the assistance of coaches that have received specific training to facilitate the change process, elicit motivation, and build trust, self-efficacy, and growth-promoting relationships. It is appropriate for type 2 diabetes management given that the coaching model is intended to address psychosocial factors and lifestyle behaviors[11]. Health coaching interventions "target health behavior changes aligned with self-determined goals leading to improved physical and mental health outcomes"[12,13]. Motivational interviewing is a patient-centered communication technique that involves open-ended questions, reflective listening, and support for patient autonomy and self-efficacy with aims to evoke intrinsic motivation of an individual to make behavior changes[14]. The objective of this review is to summarize interventions that have significantly improved knowledge, motivation, attitude, practice, and outcomes of type 2 diabetes patients.

Health education interventions

Education on knowledge, attitude, and practice: A study has reported that a health education intervention had a positive impact on the knowledge, attitude, and practice of individuals with type 2 diabetes[15].

A health education intervention consisting of 18 sessions for South Asian diabetes patients in Scotland significantly improved the low baseline scores for knowledge (+ 12.5%), serious attitudes toward diabetes (+ 13.5%), and practice (+ 20.0%)[15].

Education on knowledge, attitude, practice, and outcomes: Studies have reported that health education interventions had a positive impact on the knowledge, attitude, practice, and outcomes of individuals with type 2 diabetes[16-18].

A pharmacist-provided patient counseling in India on patients' perceptions about disease management and QoL improved knowledge, attitude, and practices scores; reduced mean capillary blood glucose levels; and improved mean scores for QoL[16].

A counseling intervention for patients during monthly sessions lasting 20-25 min for three months in South India significantly improved KAP scores, especially knowledge and attitude, and improved the outcome for postprandial blood glucose levels. However, no significant improvements in practice were reported due to high baseline scores[17].

A meta-analysis found that self-management education continually improves the outcome of HbA1c levels and suggested that knowledge and attitude continue to influence practice and outcome after the educational interventions are over[18].

Education on knowledge and outcomes: Studies have reported that health education interventions had a positive impact on the knowledge and outcomes of individuals with type 2 diabetes[19,20].

A systematic review of 72 studies evaluating the effectiveness of self-management education lasting for a period of six months or less concluded that such interventions significantly improve knowledge and glycemic control while having variable effects on lipids[19].

A community, pharmacy-based diabetes education intervention based on the American Diabetes Association standards in the United States improved knowledge of diabetes, HbA1c levels, fasting blood glucose levels, lipid levels, and blood pressure measurements[20].

Education on attitude: A study has demonstrated that a health education intervention had a positive impact on the attitude of individuals with type 2 diabetes[21].

Diabetes group education for urban, newly diagnosed patients in Ireland continually improved the patients' attitudes about the seriousness of the condition over time[21].

Education on practice and outcomes: A study has reported that a health education intervention had a positive impact on the practice and outcomes of individuals with type 2 diabetes[22].

During a health education intervention that involved access to interactive, self-paced web-based tutorials supplemented with a printout, changes in the practices of healthy eating, physical activity, blood sugar monitoring, blood pressure monitoring, foot care, and avoidance of smoking were associated with significant improvements in the outcome of HbA1c levels[22].

Education on outcomes: Studies have reported that health education interventions had a positive impact on the outcomes of individuals with type 2 diabetes[9].

A systematic review of 118 unique interventions, which involved various elements to improve participants' knowledge, skills, and ability to perform self-management activities as well as informed decision-making around goal setting, found data demonstrating that engagement in diabetes self-management education significantly decreases HbA1c levels[9].

Health coaching interventions

Coaching on attitude and outcomes: A study has reported that health coaching interventions had a positive impact on the attitude and outcomes of individuals with type 2 diabetes[11].

An integrative health coaching intervention in the United States consisting of fourteen 30-min sessions conducted over the phone, which focused on individualized visions of health and self-chosen goals, significantly decreased perceived barriers to medication adherence and improved patient activation, perceived social support, benefit finding, and HbA1c levels[11].

Coaching on practice: A study has reported that health coaching interventions had a positive impact on the practice of individuals with type 2 diabetes[23].

A three-month peer-led self-management coaching program that involved three monthly home visits and follow-up contacts through phone and email for recently diagnosed patients in the Netherlands improved the self-efficacy in intervention group patients with low baseline self-efficacy[23].

Coaching on practice and outcomes: Studies have reported that health coaching interventions had a positive impact on the practice and outcomes of individuals with type 2 diabetes[24,25].

A six-month coaching intervention in Australia consisting of monthly phone-based coaching sessions to establish and assess progress toward individualized goals for self-care activities (*e.g.*, diet, activity) and monitoring (*e.g.*, foot and eye care, vaccinations) in addition to usual care significantly improved the practices of physical activity and adherence to monitoring exams for complications, as well as the outcomes of HbA1c levels, fasting glucose, and diastolic blood pressure[24].

A 16-mo health coaching intervention at outpatient clinics in Turkey which included five or six in-person meetings and three or four telephone coaching sessions significantly improved the practice of oral health and outcome of HbA1c levels, particularly among high-risk patients, compared to formal health education[25].

Coaching on outcomes: Studies have reported that health coaching interventions had a positive impact on the outcomes of individuals with type 2 diabetes[12,26,27].

A health coaching intervention in Canada which involved weekly communication with a health coach either in-person, using a mobile device, or using a web-based wellness platform to promote goal setting and monitor progress, as well as access to a free community exercise center, improved the outcomes of HbA1c levels at three months and of body weight and waist circumference at three and six months[12].

A healthcare provider-mediated, remote coaching system *via* a PDA-type glucometer and the Internet in South Korea significantly reduced HbA1c levels (8.0% *vs* 7.5%) and total cholesterol (10.7 mmol/L *vs* 10.4 mmol/L) at three-month follow-up[26].

A clinic-based peer health coaching intervention for low-income patients with poorly controlled diabetes in the United States significantly decreased HbA1c levels to under 7.5% for 22.0% of coached mmol/L 14.9% of usual care patients at five months[27].

Motivational interviewing interventions

Motivation on knowledge and attitude: A study has reported that motivation had a positive impact on the knowledge and attitude of individuals with type 2 diabetes[28].

Training for general practitioners in motivational interviewing in Denmark significantly impacted the patients in the intervention group, who became more autonomous and motivated in their inclination to change behavior, more conscious of the importance of controlling their diabetes, and had a significantly better understanding of the ability to prevent complications[28].

Motivation on practice: Studies have reported that motivation had a positive impact on the practice of individuals with type 2 diabetes[29-31].

A 12-mo motivational interviewing-based personalized program in the United Kingdom significantly improved healthy eating habits[29].

Motivational interviewing counseling sessions for newly diagnosed patients in the Netherlands significantly improved the practice of healthy eating by reducing saturated fats[30].

A three-month motivational interviewing-based information-motivation-behavioral skills intervention for type 2 diabetes patients in the United States significantly improved the practice of healthy eating[31].

Motivation on practice and outcomes: Studies have reported that motivation had a positive impact on

the practice and outcomes of individuals with type 2 diabetes[32-37].

A 16-wk motivational interviewing intervention, in addition to a behavioral weight control program, for obese female patients in the United States significantly improved treatment adherence through higher attendance at group meetings, increased diary entries, better blood glucose monitoring, and the outcome of HbA1c levels[32].

A motivational interviewing intervention, in addition to an 18-mo weight management program, for overweight and uncontrolled female patients in the United States significantly improved treatment adherence through higher attendance at group meetings and increased diary entries, as well as the outcomes of weight loss and HbA1c levels[33].

A three-month motivational interviewing intervention in Taiwan that involved a 45- to 60-min interview, in addition to hospital-based educational sessions and the hospital's support group for diabetes patients, significantly improved patients' self-management, self-efficacy, QoL, and HbA1c levels[34].

A 13-wk motivational interviewing-based eating behavior modification program for obese patients in Thailand significantly improved the practice of healthy eating, HbA1c levels, and BMI[35].

A six-month motivational interviewing intervention with 30-min monthly sessions focusing on behavior change in an outpatient clinic after discharge for hospitalized patients with poor long-term glycemic control in China significantly improved the practice of self-management and the outcome of homeostatic model assessment for insulin resistance scores[36].

A motivational interviewing intervention for African American adults in the United States significantly increased the odds of participants adhering to recommended physical activity level (66.7% *vs* 38.8%) and significantly decreased glucose levels and BMI[37].

Motivation on outcomes: Studies have reported that motivation had a positive impact on the outcomes of individuals with type 2 diabetes[38,39].

A motivational interviewing intervention and a cognitive behavioral group training intervention each consisting of four 90-min sessions in Iran significantly lowered the mean BMI[38].

A two-year motivational interviewing-based behavior change counseling program for high-risk patients in the United States significantly improved blood pressure[39].

DISCUSSION

The empirical literature illustrates the beneficial impacts of interventions involving self-management education, health coaching, and motivational interviewing for diverse type 2 diabetes patients. This review contributes to the understanding of the KMAP-O framework and how it can guide the care management of patients with type 2 diabetes. It will allow the tailoring of interventions to be more effective through knowledge enhancement, increased motivation, attitudinal changes, and improved preventive practice to reduce the progression of type 2 diabetes and comorbidities. To ensure the effectiveness of such interventions, outcome tracking can be conducted through longitudinal observations of patients and their knowledge, motivation levels, attitude, practice, and outcomes.

Multiple clinical symptoms such as low plasma adiponectin[40], obesity and sarcopenia[41], and defective fat oxidation capacity[42] are linked with type 2 diabetes. Thus, clinical interventions should be designed carefully through causal specifications of the etiology of type 2 diabetes. Obesity is considered a leading cause for type 2 diabetes and cardiovascular disease. It is concluded that diabetes care should not only pay attention to clinical symptoms or etiologies associated with diabetes, but also consider behavioral factors that could either impede or facilitate patient adherence and self-care management of a controllable chronic condition. Furthermore, the efficacy of health promotional strategies, using the KMAP-O framework, can be demonstrated by carefully designed and executed clinical trial studies that are augmented with health information technology[43].

COMMENTS

Background

This manuscript summarized the relevance of behavioral components of health education that can improve diabetes care. Type 2 diabetes is considered an ambulatory care sensitive condition. Proper implementation of care management strategies can prevent unnecessary hospital admissions and readmissions.

Research frontiers

This manuscript introduced a comprehensive model grounded by behavioral and social science theories through a careful review of the scientific literature and document relevant strategies for implementing diabetes education and achieving diabetes control.

Innovations and breakthroughs

This manuscript articulated the potential causal mechanisms for enhancing preventive practice and improving patient care outcomes of type 2 diabetes.

Applications

This manuscript suggested plausible causal links between health educational intervention and patient care outcomes mediated by behavioral factors such as knowledge, motivation, attitude, and practice relevant to diabetes care.

Peer-review

This manuscript is a literature review on impacts of interventions involving self-management education, health coaching, and motivational interviewing for diverse type 2 diabetes patients.

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REVIEW

Effects of glycaemic management on diabetic kidney disease

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Abstract

Hyperglycaemia contributes to the onset and progression of diabetic kidney disease (DKD). Observational studies have not consistently demonstrated a glucose threshold, in terms of HbA1c levels, for the onset of DKD. Tight glucose control has clearly been shown to reduce the incidence of micro- or macroalbuminuria. However, evidence is now also emerging to suggest that intensive glucose control can slow glomerular filtration rate loss and possibly progression to end stage kidney disease. Achieving tight glucose control needs to be balanced against the increasing appreciation that glucose targets for the prevention of diabetes related complications need be individualised for each patient. Recently, empagliflozin which is an oral glucose lowering agent of the sodium glucose cotransporter-2 inhibitor class has been shown to have renal protective effects. However, the magnitude of empagliflozin's reno-protective properties are over and above that expected from its glucose lowering effects and most likely largely result from mechanisms involving alterations in intra-renal haemodynamics. Liraglutide and semaglutide, both injectable glucose lowering agents which are analogues of human glucagon like peptide-1 have also been shown to reduce progression to macroalbuminuria through mechanisms that remain to be fully elucidated. Here we review the evidence from observational and interventional studies that link good glucose control with improved renal outcomes. We also briefly review the potential reno-protective effects of

newer glucose lowering agents.

Key words: Diabetic nephropathy; Albuminuria; Glucose control; Glomerular filtration rate; Diabetes; Chronic kidney disease; Empagliflozin; Liraglutide; Semaglutide

Core tip: Tight glucose control has been clearly shown to reduce the incidence of micro- and macroalbuminuria. Evidence is now also emerging to suggest that intensive glucose control can slow glomerular filtration rate loss and possibly progression to end stage kidney disease. Furthermore, empagliflozin which is a glucose lowering agent of the sodium glucose like transporter-2 inhibitor class has been shown to have reno-protective effects over and above those expected from its glucose lowering effects alone. Recent clinical trials have also shown that Liraglutide and Semaglutide, injectable glucose lowering agents which are analogues of human glucagon like peptide-1, reduce progression to macroalbuminuria.

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INTRODUCTION

Diabetic kidney disease (DKD) is the most common cause of end-stage kidney disease (ESKD). People with DKD are not only at significant risk of progression to ESKD but have a greater concomitant increase in the risk for cardiovascular (CV) morbidity and mortality[1]. Therefore, optimising treatments to prevent the development and progression of DKD are of the utmost importance.

DKD does not occur in the absence of hyperglycaemia and glucose control is the main determinant of the onset of nephropathy. Despite this, the role of optimising glucose control in slowing the progression of DKD still remains controversial, but evidence is now emerging to suggest that intensive glucose control can slow glomerular filtration rate (GFR) loss and possibly progression to ESKD. Indeed, there is now evidence from observational studies that that good glucose control, at least in patients with type 1 diabetes (T1DM), is associated with improved kidney health even in the setting of advanced DKD[2-4].

Some of the best evidence supporting the beneficial effects of tight glucose control on the kidneys of people with T1DM and advanced nephropathy has come from studies involving patients that have received a combined pancreatic and kidney transplant. Near complete reversal of many of the structural parameters associated with classical diabetic nephropathy has been observed in the native kidneys of these patients after 10 years of normoglycaemia[5].

Here we review the results of observational and interventional studies that have examined the effects of glycaemia on markers of kidney health in people with diabetes. We also briefly review the potential reno-protective effects of newer glucose lowering agents, especially the sodium glucose cotransporter-2 (SGLT-2) inhibitors which have recently been shown to reduce the chances of high risk vascular patients with type 2 diabetes progressing to clinically meaningful renal endpoints. A review of glycaemic management and the optimum way to assess glycaemic control in ESKD patients is beyond the scope of this review. We have also not reviewed the impact of failing kidney function on glucose and insulin metabolism.

MEASURING DKD OUTCOMES IN CLINICAL TRIALS

Studies examining the effect of glycaemia on the development and progression of DKD have usually focused on three outcome parameters. One parameter is changes in albuminuria, with a transition from micro- to macroalbuminuria occurring in the majority of patients with diabetes who develop ESKD. It is now firmly established that good glucose control can prevent the onset and progression of albuminuria. However, the entities of normoalbuminuric and non-proteinuric renal insufficiency are increasingly being recognised and the specificity of microalbuminuria as a marker for progressive diabetic renal disease remains to be established. These findings call into question the reliability of albuminuria, especially within the microalbuminuric range, as a surrogate kidney health outcome[6].

The second parameter is changes in creatinine or GFR. Caution is also required in interpreting the significance of these outcomes. Under certain circumstances, a rise in serum creatinine, and hence a decrease in estimated GFR (eGFR), may represent transient changes in kidney perfusion or function which are not necessarily related to the causal pathway for the development of chronic kidney disease (CKD)[7].

Lastly, the development of "hard-renal endpoints", death due to renal disease and/or the development of ESKD are the outcomes that ideally should be the primary endpoint of most renoprotective trials. From a practical point of view, end-points such as these are usually only worth considering in large, long-term trials in high risk patients with sufficient rates of events to allow statistical comparisons to be made.

In recent years, tight glucose control had been shown to slow GFR decline in subjects with T1DM in the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes and Interventions and Complications (EDIC) study and to slow progression to ESKD in subjects with type 2 diabetes (T2DM) in the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) study and its follow up observational study, ADVANCE ON[7-9].

MOLECULAR EFFECTS OF HYPERGLYCAEMIA ON THE DIABETIC KIDNEY

The cellular mechanisms responsible for hyperglycaemia mediated renal damage include accelerated generation of advanced glycation end products and activation of their receptor (RAGE), which triggers increases in protein kinase protein kinase C, nuclear factor-kappa B, transforming growth factor-β and connective tissue growth factors[10]. The resultant generation of reactive oxygen species (ROS) and a chronic subacute inflammatory process play a pivotal role in the development of DKD.

In addition, RAGE activation induces glomerular matrix production and increases oxidative stress through mitochondrial superoxide production[11]. Studies in experimental diabetes have also shown that RAGE activation promotes epithelial-mesenchymal transdifferentiation of renal tubular cells, thereby contributing to interstitial fibrosis[12]. Recent studies have also demonstrated that RAGE medicated mitochondrial dysfunction, most likely due to activation of NAD(P)H oxidase (NOX), an enzyme dedicated to the production of ROS, is an early manifestation of DKD and precedes the development of albuminuria and renal histological change[13]. Despite these promising experimental studies like those mentioned above, clinical trials to date have failed to establish that non-glucose lowering based approaches that specifically target pathways linked to oxidative stress or inflammation are renoprotective. Therefore, in recent years there has been renewed interest in robustly establishing the beneficial effects of tight glucose control for reducing the development and progression of DKD.

OBSERVATIONAL STUDIES

Pre diabetes

Even before the glucose thresholds for the diagnosis of diabetes are reached, elevated glucose levels have been associated with an increasing prevalence of CKD. A systematic review and meta-analysis has recently examined the relationship of prediabetes (impaired fasting glucose and/or impaired glucose tolerance) and the incidence of CKD. In an analysis involving eight studies with samples sizes ranging between 2398 and 118924, prediabetes was found to be modestly associated with an increased risk for CKD compared to normal glucose tolerance after adjustment for established risk factors with a RR of 1.11(95%CI: 1.02-1.21)[14]. Higher HbA1c levels have been shown to be associated with lower eGFR values in 24594 South Korean Adults without a history of diabetes and with all subjects used in the analysis having a HbA1c level < 6.5% (48 mmol/mol). The association between HbA1c levels and CKD in this study was evident in subjects with and without the metabolic syndrome[15].

Diabetes

The DCCT in T1DM and the United Kingdom Prospective Diabetes Study (UKPDS) in T2DM have both demonstrated a strong relationship between glucose control and the risk of the development of diabetic microvascular complications without a clear-cut HbA1c threshold[16,17]. The rate of progression of albuminuria in patients (both with T1DM and T2DM) who developed persistent microalbuminuria has also been shown to correlate with the degree of long-term glucose control over approximately 12 years of follow-up[18].

However, a sophisticated observational analysis from the ADVANCE study, involving subjects with T2DM, suggests that within the range of HbA1c studied [5.5%-10.5% (37-91 mol/mol)], there was evidence of a glucose threshold such that below HbA1c levels of 6.5% (48 mmol/mol) there was no significant change in the risks for the development of eye or kidney complications including macroalbuminuria, doubling of serum creatinine levels, need for renal-replacement therapy, or death due to renal disease[19].

In contrast, an observational sub-study of the Outcome Reduction with Initial Glargine Intervention trial that involved 12537 people with T2DM or prediabetes followed for 6.2 years suggested that the risk for adverse renal outcomes rises progressively from the lowest [< 5.7% (33.8 mmol/mol)] to the highest quintile [7.4% (57.4 mmol/mol)] of HbA1c levels. In this study, the HR for renal failure was 1.54 (95%CI: 1.24-1.91, P = 0.001) per 1% higher baseline HbA1c[20]. In the Atherosclerosis Risk in Communities study involving 1871 adults with diabetes (presumed T2DM in the vast majority) followed for 11 years, there was a graded relationship between higher HbA1c levels and incident CKD (defined as an eGFR < 60 mL/min per 1.73 m^2) that was independent of traditional risk factors and present even in the absence of albuminuria and retinopathy. However, a significant increase in risk for the development of CKD was only evident for HbA1c values greater that 7% (53 mmol/mol)[21].

A large population-based cohort study has also shown an increased risk of ESKD with higher HbA1c levels. In this study, 23296 patients with stage 3 or 4 CKD were identified and then followed for approximately 4 years using laboratory data, hospitalisation and insurance claims. Patients were then stratified by glucose control based on the first HbA1c measured during the study period. The median baseline HbA1c was 6.9% (52 mmol/mol) and 11% had levels > 9% (75 mmol/mol). During the study, 16% patients died, 16% had a cardiovascular event and 6% progressed to ESKD. In an analysis adjusted for age, sex, GFR, socio-economic factors and co-morbidities, an increased risk of ESKD was associated with higher HbA1c levels but this relationship was attenuated in patients with a lower eGFR[22].

Among patients with stage 3 CKD (eGFR 30-59 mL/min per 1.73 m^2), the risk for ESKD was increased by 22% for those with HbA1c levels between 7 and 9% (53 and 75 mmol/mol) and by 152% for those with levels > 9% (75 mmol/mol), compared to those with a HbA1c < 7% (53 mmol/mol). In contrast, for patients with stage 4 CKD (eGFR 15-29 mL/min per 1.73 m^2) at baseline, the corresponding increases in risk for ESKD were only

3% and 13%, respectively, compared with patients with a HbA1c < 7% (53 mmol/mol). Interestingly, the severity of CKD was not a significant modifier of the relationship between glucose control and a doubling of serum creatinine. However, it is worth noting that the use of doubling of serum creatinine in isolation as a renal endpoint has been questioned recently. It was suggested by the authors that the relationship between HbA1c and ESKD was stronger in patients with milder CKD as there may be a threshold of kidney function below which better glucose control alone in not enough to prevent progressive kidney loss.

Interestingly, in the above study, a U-shaped relationship was found between glucose control and total mortality, with increases in the risk of mortality apparent at HbA1c levels < 6.5% (48 mmol/mol) and greater than 8.0% (64 mmol/mol). The nature of the relationship between HbA1c and mortality was different from that between HbA1c and other outcomes such as ESRD, hospitalization, and cardiovascular events. Furthermore, the relationship between HbA1c and outcomes, apart from that of ESKD, was not modified by initial GFR level or stage of CKD. This result together those of the already mentioned observational study from ADVANCE suggest that ideally a HbA1c threshold of 6.5% (48 mmol/mol) should be targeted as a means of preventing the development and progression of DKD[19]. However, the importance of individualising glycaemic targets according to a patient's age, co-morbidities and type of glucose lowering therapies prescribed is appreciated.

Two observational studies from the Joslin Diabetes Clinic, Boston, United States, have also highlighted the importance of the relationship between glucose control and progression of kidney disease in people with diabetes. In the first study, patients with T1DM that experienced an early progressive decline in GFR, defined as a GFR loss over and above that expected with aging alone that starts before a GFR threshold of 60 mL/min per 1.73 m^2 is reached, were identified from a cohort with microalbuminuria[23]. For the 301 patients studied, 207 had stable renal function and 94 had declining renal function over 8 to 12 years of observation. Patients with a baseline HbA1c $\geqslant$ 9% (75 mmol/mol) had an odds ratio of 2.5 (95%CI: 1.2-5.4) for having an early progressive decline in GFR compared with patients with an initial HbA1c < 9.0% (75 mmol/mol). This early decline in GFR was believed to be distinct from resolution of hyperfiltration, because it was progressive, occurred in patients with relatively long duration of diabetes (approximately 18 years), and because improved, and not worsening glucose control, has been associated with resolution of hyperfiltration.

The second study from the Joslin Diabetes Clinic suggests that sustained improvement in glucose control in T1DM patients with overt proteinuria can reduce the long-term risk of ESKD. A 1% improvement in HbA1c over a follow-up period of approximately 3.5 years was associated with a HR for ESKD of 0.72 (95%CI: 0.61-0.89) which was independent of other covariates[24].

Pancreatic transplant studies

Some of the most impressive information supporting the beneficial effects of tight glucose control on the kidneys of people with T1DM has come from a study involving subjects with DKD who received a combined pancreatic and kidney transplant. After 5 years of normoglycaemia, achieved by pancreatic transplantation, biopsy specimens of the transplant recipient's native kidneys showed no change in kidney structure. However, after 10 years of normoglycaemia, there was remarkable remodelling of kidney structure, with near complete reversal of many of the structural parameters associated with classical diabetic nephropathy[25].

Glucose and HbA1c variability

There is no convincing evidence that short term (within-day) variability in glucose levels influences the risk for the development of DKD. A substudy from the DCCT showed that within-day glucose variability, as assessed by seven-point laboratory measured glucose levels, did not influence the risk for the development of nephropathy over and above that conferred by mean glucose levels alone[26].

In contrast, numerous studies have demonstrated that long-term glucose variability as assessed by HbA1c measurements is associated with the risk for the development and progression of DKD. In patients with T1DM followed for approximately 6 years, the standard deviation of serial HbA1c measurements has been shown to be associated with the risk of progressive renal disease (HR = 1.94, 95%CI: 1.49-2.47) as assessed by progression of albuminuria or the development of ESKD, even after adjustment for mean HbA1c and traditional risk factors[27].

In patients with T2DM, variability of HbA1c measurements over 2 years period before enrollment in the Renal Insufficiency and Cardiovascular Events Italian Multicentre study was found to be independently related to the prevalence of albuminuria and reduced GFR assessed during the subsequent 2 years of the study. The variably in HbA1c measurements in the preceding 2 year period was found to be more strongly associated with the prevalence of DKD than average HbA1c levels. In contrast, average values but not variability in HbA1c was associated with the prevalence of retinopathy[28]. The above results suggest that variability as opposed to mean HbA1c levels may be associated with the development and progression of diabetic microvascular complications *via* different but as yet, unexplained mechanisms.

INTERVENTIONAL STUDIES

In-trial results

In the DCCT, subjects with T1DM were randomised to intensive glucose control [HbA1c 7.3% (56 mmol/mol), *n* = 711] or conventional glucose control [HbA1c 9.1% (76 mmol/mol), *n* = 730] and studied for 6.5 years. At the end of the intervention, intensive therapy was associated with a significant reduction in the number

of subjects that developed microalbuminuria (10.2 *vs* 17.7%, respectively, $P < 0.01$) or macroalbuminuria (1.4 *vs* 3.2, respectively, $P < 0.05$). Estimated GFR decreased in the intensive glucose control group (from 126 to 121.8 mL/min per 1.73 m^2) during the first year of the trial, a decrease that was 1.4 mL/min per 1.73 m^2 ($P < 0.001$) greater than that in the conventional glucose control group. Thereafter GFR declined in parallel for the two treatment groups. This initial drop in estimated GFR in the intensive glucose control group most likely presented a resolution of hyperfiltration. Due a low event rate it was not possible to assess the effects of good glucose control on ESKD rates in the DCCT[16].

In the UKPDS, subjects with T2DM were randomised to intensive glucose control [HbA1c 7.0% (53 mmol/mol), $n = 2408$] or conventional glucose control [HbA1c 7.9% (63 mmol/mol), $n = 994$] and studied for 10 years. Nine years into the intervention, intensive therapy was associated with a significant reduction in the number of subjects that developed microalbuminuria (19% *vs* 25%, respectively, $P < 0.001$) or macroalbuminuria (4.4% *vs* 6.5%, respectively, $P = 0.026$). No differences in ESKD rates were reported in the trial but the number of patients experiencing a doubling of serum creatinine 9 years were lower in the intensive therapy *vs* the conventional therapy group, although it should be noted that event rates in both groups were very low (0.71% *vs* 1.76%, $P = 0.027$)[29].

In the ADVANCE study, 11140 subjects with T2DM were randomised to intensive glucose control or conventional glucose control and followed for a median of 5 years. The time weighted average HbA1c difference was 0.67% for intensively [mean HbA1c 6.5% (48 mmol/mol) at the end of the study] and conventionally treated subjects [HbA1c 7.3% (56 mmol/mol) at the end of the study]. The majority of subjects in the study had normal kidney function but, 19% had a GFR < 60 mL/min per 1.73 m^2, 27% had microalbuminuria and 4% had macroalbuminuria. The ADVANCE study originally reported that intensive glucose control reduced the risk of the composite endpoint of new or worsening nephropathy, comprising the composites of new-onset macroalbuminuria, ESKD, renal death or doubling of serum creatinine to > 200 μmmol/L) by 21% (HR = 0.79, 95%CI: 0.66-0.93, $P = 0.006$) compared to conventional glucose control[30].

Due to concerns about the reliability of changes in albuminuria and creatinine as surrogate endpoint of kidney health a new analysis of the ADVANCE data was performed to specifically examine the effects of intensive glucose control on clinical kidney health outcomes such as the development of ESKD. In this study, the risk of ESKD was 65% lower in subjects randomised to intensive compared with conventional glucose control (HR = 0.35, 95%CI: 0.15-0.83, $P = 0.01$). This result was still seen after considering the confounding influences of renal or all-cause death. Fewer subjects randomised to intensive glucose control had a sustained doubling of serum creatinine compared to conventional glucose control but this outcome failed to reach statistical significance (HR = 0.87, 95%CI: 0.54-1.27). Subjects with a sustained doubling of serum creatinine were restricted to those with a final creatinine measurement that was still above the doubling threshold[7].

However, it should be noted that two other contemporary intensive glucose control trials in subjects with T2DM have failed to demonstrate that tight glucose control significantly attenuates a decline in GFR and the development of ESKD. In the Action to Control Cardiovascular Risks in Diabetes (ACCORD) study the risk for ESKD was 5% lower with intensive glucose control (HR = 0.95, 95%CI: 0.73-1.24, NS) but doubling of serum creatinine or a 20 mL/min per 1.73 m^2 decrease in estimated GFR risk was 7% higher with intensive glucose control (HR = 1.07, 95%CI: 1.01-1.13, $P = 0.016$)[31]. The limitations of using single creatinine measurements as an outcome parameter have been already discussed. In the Veterans Affairs Diabetes Trial (VADT), rates of decline in GFR and the achievement of an estimated GFR < 15 mL/min per 1.73 m^2 were not different with intensive or conventional glucose control. Although intensive glucose control had no significant effect on estimated GFR decline in the whole VADT cohort, it did slow estimated GFR decline in subjects with high baseline levels of albuminuria (OR = 0.61, 95%CI: 0.37-1.00, $P = 0.04$)[32]. The effects of intensive glucose control on renal outcomes in trials involving participants with T2DM are summarised in Table 1.

Meta-analysis results

A Cochrane review punished in 2014 examined the potential benefits of intensive *vs* conventional glucose control in T1DM. Whist intensive glucose control was found to reduce the risk for the development of microalbuminuria for 1475 participants in 3 trials (RR = 0.56, 95%CI: 0.46-0.68, $P < 0.00001$). There was no significant reduction in progression to macroalbuminuria (RR = 0.79, 95%CI: 0.37-1.70). Insufficient trial participants reached ESKD for any meaningful analysis of the benefits of intensive glucose control on this outcome[33].

In contrast, a very recent systemic review and meta-analysis of 5 trials that included information on 1635 participants with T1DM has shown that nephropathy (defined as albumin excretion rate > 300 mg/24 h or using an outcome of "clinical nephropathy") is reduced with intensive glucose control compared with conventional glucose control (RR = 0.37, 95%CI: 0.27 to 0.50; $P < 0.00001$). Not unexpectedly, due to low event rates, the effect of intensive glucose targets on ESKD rates was not statistically significant (RR = 0.96, 95%CI: 0.13-7.05, 3 trials, 124 participants)[34].

A meta-analysis of intensive glucose control studies in T2DM by Coca *et al*[35], that included results from the UKPDS, ADVANCE and ACCORD trials and evaluated outcomes in 28065 patients with type 2 diabetes over 2 to 15 years, has shown that compared to conventional control [median HbA1c values 7.3%-9.4% (56-79 mmol/mol)], intensive glucose control [median HbA1c 6.4%-7.4% (46-57 mmol/mol)] reduced the risk for development of microalbuminuria (RR = 0.86, 95%CI: 0.76-0.96) and macroalbuminuria (RR = 0.74, 95%CI: 0.65-0.85).

Table 1 Baseline characteristics and renal end points of randomised trials of intensive *vs* standard glucose control in type 2 diabetes

	UKPDS[29]	ACCORD[30,31]	ADVANCE[7,30]	VADT[32]
Baseline characteristics				
No. of subjects	3867	10251	11140	1791
Age (yr)	53	62	66	60
Duration of diabetes (yr)	0	10	8	11.5
History of CV disease (%)	NR	35	32	40
Median HbA1c at baseline (%)	7.0	8.1	7.2	9.4
Duration of follow-up (yr)	10.0	3.5	5.0	5.6
Achieved median HbA1c for I *vs* S (%) (mmol/mol)	7.0 *vs* 7.9 (53 *vs* 63)	6.4 *vs* 7.5 (46 *vs* 59)	6.3 *vs* 7.0 (45 *vs* 53)	6.9 *vs* 8.5 (52 *vs* 69)
Microalbuminuria (%)	11	25	26	N/A
Macroalbuminuria (%)	2	7	4	N/A
Renal outcomes				
Microalbuminuria (HR or RR)	0.67[a] (0.53-0.86)	0.79[a] (0.69-0.90)	0.91[a] (0.85-0.98)	0.85 (0.59-1.23)
Macroalbuminuria (HR or RR)	0.66 (0.39-1.10)	0.68[a] (0.58-0.86)	0.70[a] (0.57-0.85)	0.56[a] (0.33-0.96)
Worsening albuminuria (HR or RR)	N/A	N/A	N/A	0.72[a] (0.53-0.97)
Doubling of creatinine (HR or RR)	0.26[a] (0.39-1.10)	1.07 (1.01-1.13)	0.83 (0.54-1.27)	1.0 (0.74-1.35)
Decline in eGFR (HR or RR)	N/A	N/A	N/A	0.61 (0.37-1.00)
ESKD (HR or RR)	N/A	0.95 (0.73-1.24)	0.35[a] (0.15-0.83)	0.63 (0.25-1.6)

[a]Significant reduction with intensive glucose control. CV: Cardiovascular; eGFR: Estimated glomerular filtration rate; ESKD: End stage kidney disease; HR: Hazard ratio; RR: Relative risk; N/A: Not available; HbA1c: Glycosylated hemoglobin; I: Intensive glucose control; S: Standard glucose control; UKPDS: United Kingdom Prospective Diabetes Study; ACCORD:Action to Control Cardiovascular Risk in Diabetes; ADVANCE: Action in Diabetes and Vascular Disease Pretrax and Diamicron Modified Release Controlled Evaluation; VADT: Veterans Administration Diabetes Trial.

Although the risk for the development of ESKD was estimated to be reduced by 31% (RR = 0.69, 95%CI: 0.45-1.05) by this analysis, this reduction with intensive glucose lowering was not significantly different compared with conventional glucose control. This result was possibly related to the relatively low incidence of ESKD (< 1.5%) compared with that of microalbuminuria (23%) and macroalbuminuria (5%).

Another Cochrane review published in 2012 also examined the potential benefits of intensive *vs* conventional glucose control in T2DM and reported that the risk of progressive nephropathy (defined as the development of macroalbuminuria, renal failure, doubling of creatinine or reduced GFR in 27929 participants in 9 trials) was reduced by intensive glucose control (RR = 0.78, 95%CI: 0.61-0.99, $P < 0.04$). As above, intensive glucose control was associated with a non-significant reduction in the risk for ESKD for 28075 participants in 7 trials (RR = 0.87, 95%CI: 0.72-1.07)[36].

Post-trial follow-up results

Although there is good evidence to suggest that elevated glucose levels are an important initiator and promoter of early DKD and interventions to improve glycaemia can slow the progression of early DKD, as discussed above, there has been only limited evidence to suggest that improving glycaemia slows the rate of GFR decline and retards progression to ESKD. However, evidence from very recent studies suggests that improving glucose levels may ameliorate a decline in GFR and progression to ESKD (Table 2).

A study from the DCCT/EDIC Research group has shown that the long term risk for the development of an impaired GFR (< 60 mL/min per 1.73 m^2) was significantly lower for subjects with T1DM who were initially treated to achieve tight glucose control[8]. In this study, participants were followed for 6.5 years during randomisation to intensive or conventional glucose control arms of the DCCT and then followed up for a further 16 years in the EDIC study. Of note, the approximate 2% difference in HbA1c levels in the intensive compared to conventional glucose control arms of DCCT was no longer apparent at the end of the EDIC study with Hb1Ac levels being approximately 8.0% (64 mmol/mol) for all subjects.

At the end of a further 16 year observational study, less subjects who were originally randomised to intensive glucose control developed macroalbuminuria (3.2 *vs* 7.3, $P < 0.01$). However, there was no difference in the rate of development of microalbuminuria[8,16]. The risk for developing a sustained impairment of GFR was approximately 50% lower in subjects originally randomised to intensive glucose control in the DCCT compared to conventional glucose control. However, this effect was not evident until 10 years after the completion of randomisation to *vs* conventional intensive glucose control. In this study, the outcome of a sustained impairment of GFR was based on the finding of two consecutive annual estimates of GFR. ESKD developed in only 8 subjects randomised to intensive glucose control compared with 16 subjects randomised to conventional glucose control. However, due to the low number of subjects that developed ESKD the difference in this event rate was not statistically significant. In contrast, initial intensive glucose control reduced the risk of impaired GFR or death by 37% (95%CI: 10-55, $P = 0.01$).

After the end of the randomised component of the UKPDS, participants were followed up for a further 10 years to assess the development of microvascular complications. Any difference in HbA1c levels between the intensive and conventional glucose control groups was lost after one year of follow-up. Despite this, the significant 24% reduction in the risk for microvascular complications

Table 2 Renal end points in observational post-randomisation trials of intensive glucose control in type 1 and type 2 diabetes

	DCCT/EDIC	ADVANCE ON
Diabetes type	1	2
n	1375	8494
Impaired eGFR (HR or RR)	0.63[a] (0.10-0.55)	N/A
Impaired eGFR or death (HR or RR)	0.5[a] (0.18-0.69)	N/A
ESKD (HR or RR)	0.49 (-0.14-0.79)	0.54[a] (0.34-0.85)
ESKD or renal death (HR or RR)	0.63 (0.10-0.55)	N/A

[a]Significant reduction with intensive glucose control. DCCT/EDIC: Diabetes control and complications trial/epidemiology of diabetes and interventions and complications; ADVANCE: Action in diabetes and vascular disease: Pretrex and diamicron MR controlled evaluation; eGFR: Estimated glomerular filtration rate; ESKD: End stage kidney disease; HR: Hazard ratio; RR: Relative risk; N/A: Not available.

at the end of the intervention trial was sustained during the post-trial observational period. However, during the 10-year observational period, subjects originally randomised to intensive or conventional glucose control had similar levels of albuminuria and serum creatinine levels, suggesting that the reduction in microvascular complications was almost entirely accounted for by lower rates of eye related events[37].

In a similar fashion to the follow-up that occurred in the UKPDS, after the completion of the randomisation component of the ADVANCE trial, patients were followed for a further 5.4 years. Any difference in HbA1c levels disappeared by the first post-trial visit and values remained at around 7.3% (56 mmol/mol) during the observational component of the study. During the total follow period of 9.9 years, the significant reduction in ESKD during the in-trial period (7 *vs* 20 events, HR = 0.35, 95%CI: 0.15-0.83, $P = 0.02$) persisted, but the HR was attenuated (29 *vs* 53 events, HR = 0.54, 95%CI: 0.34-0.85, $P < 0.01$). It was suggested that over the 9.9 years of the study, 194 participants would need to be treated with intensive glucose control to revent one ESKD event.

The renal results from ADVANCE-ON suggest that intensive glucose control may only be of benefit before the onset of clinically relevant CKD. Subgroup analysis demostrated a significant heterogenisity for the risk of ESKD with intensive glucose control according to baseline CKD stage: No CKD (HR = 0.16, 95%CI: 0.04-0.74), CKD stages 1 and 2 (HR = 0.34, 95%CI: 0.12-0.95) and CKD stges $\geqslant$ 3 (HR = 0.89, 95%CI: 0.47-1.67). Furthermore, for participants with an eGFR greater than or equal to 60 mL/min per 1.73 m^2 compared to those with estimated GFR levels below this value, numbers needed to treat to prevent one ESKD event were reported to be 109 and 393, respectively[9]. The above result was highlihed by the authors of the ADVANCE-ON study to indicate the importance of commencing intensive glucose control in patients with type 2 diabetes before the development of established DKD.

HYPERGLYCAEMIA AND HYPERFILTRATION

Increased intra-glomerular pressure as a result of increased plasma flow and/or vasodilatation of the afferent glomerular arterioles and/or constriction of the efferent arterioles, resulting in the state of hyperfiltration is a hallmark of early DKD. One of the most important determinants of hyperfiltration is hyperglycaemia. Indeed hyperfiltration can even be induced by a state of acute hyperglycaemia, for example the elevation in glucose levels induced by a hyperglycaemic clamp. A recent study of 17 normo-filtering T1DM subjects demonstrated that GFR (measured by inulin clearance) increased from 118 mL/min per 1.73 m^2 when measured directly after an 8 h euglycaemic clamp (blood glucose 4-6 mmol/L) to 137 mL/min per 1.73 m^2 when measured after a hyperglycaemic clamp (blood glucose 9-11 mmol/L)[38].

Early hyperfiltration, occurring in the first months of T1DM has been shown to reverse with insulin therapy[39]. By contrast, late or persistent hyperfiltration may persist for years and may not be associated with glucose control when assessed by HbA1c measurements several years after the onset of diabetes. In a study involving 12 patients with T1DM and an increased GFR for a year after they were randomly assigned either to continuous subcutaneous insulin pump therapy or to unchanged conventional therapy, the glomerular filtration rate fell significantly in the pump group and became normal in four of the six patients although the kidneys remained enlarged. GFR did not change in the conventional-treatment group[40]. Therefore, these results support the theory that strict glucose control normalizes GFR, at least in the first years after the development of diabetes. The effects of improving glucose control on hyperfiltration are less well documented in patients with long standing diabetes.

The mechanism linking hyperglycaemia with the onset of hyperfiltration most likely involves an increase in sodium reabsorption *via* the SGLT-2 receptor in the proximal tubule which ultimately results in tubulo-glomerular feed-back modulating blood flow in the glomerular afferent arteriole[41]. Evidence exists in diabetic rats and humans for a primary increase in proximal tubular sodium and glucose reabsorption in the setting of hyperglycaemia. This occurs due to augmented sodium-glucose co-transport that subsequently results in a reduced sodium chloride concentration being delivered to the macula densa. This reduction in sodium chloride concentration is interpreted by the juxtaglomerular apparatus to represent a decline in circulating volume and renal perfusion. To maintain GFR, dilatation of the afferent glomerular arterioles occurs, possibly through an adenosine mediated process, which ultimately results in a state of hyperfiltration. As discussed below the renal protective effects of the SGLT-2 receptor inhibitors may be partly related to their ability to reduce intra-glomerular pressure by increasing sodium chloride delivery to the macular densa with tubulo-glomerular

Table 3 Comparison of cardiovascular outcomes and mortality in randomised trials of intensive glucose control in type 2 diabetes participants with and without chronic kidney disease

	ACCORD		ADVANCE	
	Total mortality	CV mortality	Total mortality	CV mortality
Overall study results (HR or RR)	1.22[a] (1.01-1.46)	1.35[a] (1.04-1.76)	0.93 (0.83-1.06)	0.88 (0.74-1.04)
Non-CKD participants (HR or RR)	1.08 (0.87-1.34)	1.14 (0.82-1.58)	0.74 (0.76-1.15)	0.78 (0.58-1.07)
CKD participants (HR or RR)	1.31[a] (1.07-1.60)	1.41[a] (1.05-1.89)	0.91 (0.72-1.14)	0.90 (0.67-1.22)

[a]Significant reduction with intensive glucose control. CV: Cardiovascular; CKD: Chronic kidney disease; ACCORD: Action to control cardiovascular risks in diabetes; ADVANCE: Action in diabetes and vascular disease: Pretrex and diamicron MR controlled evaluation; HR: Hazard ratio; RR: Relative risk.

feedback then resulting in a constriction of the afferent arteriole[42,43].

The relationship between SGLT-2 inhibition and the renin-angiotensin system (RAS) is complex and as yet not fully elucidated. The decrease in intraglomerular pressure and the modest volume depletion volume depletion seen with SGLT-inhibition has the potential to result in RAS activation. In contrast, the increased delivery of sodium to the macular densa may result in a reduction in RAS activation. Despite the above, SGLT-2 inhibition appears to result in an increase in RAS activity[42]. It has been suggested that increased RAS activity may in fact play an important role in maintain adequate glomerular filtration in the setting of SGLT-2 inhibition[44]. In any event, the cardiac and renal protective effects of empagliflozin appear to be consistent in patients treated or not treated with agents that block RAS activity[45,46].

RISKS AND BENEFITS OF INTENSIVE GLUCOSE CONTROL

One major concern regarding the application of intensive glucose control is the potential risk of adverse outcomes, especially patients with diabetes and CKD. As mentioned previously, randomisation to intensive glucose control in the ACCORD trial was associated with higher CV and all-cause mortality rates compared to standard glucose control. A follow-up study from ACCORD has suggested that the excess mortality associated with randomisation to tight glucose control was predominantly accounted for by participants with prevalent CKD (Stages Ⅰ-Ⅲ). The risk for the primary outcome (the first occurrence of nonfatal myocardial infarction or stroke, or cardiovascular death) for the study was 87% higher in participants with CKD compared to those without CKD (HR = 1.87, 95%CI: 1.65-2.11)[47]. In the ACCORD study rates of hypoglycaemia were also approximately twice as great in patients with CKD compared to those without CKD.

In contrast to the above, the ADVANCE-ON study results have reiterated the in trial results from ADVANCE and shown that intensive glucose control had no clear effect on all-cause mortality or cardiovascular events or death from CV disease. Importantly the results from ADVANCE-ON showed that there was no evidence that baseline CKD status (stages Ⅰ, Ⅱ and ≥ Ⅲ) had any impact on the rates of the above outcomes with intensive glucose control (Table 3).

Furthermore, as discussed in more detail later, it has recently been shown that the glucose lowering medications liraglutide and empagliflozin reduce CV events in patients with reduced GFR. Therefore, in contrast to the ACCORD study, the results of the ADVANCE-ON study and the CV safety trials involving empagliflozin and liraglutide suggest that it is possible to aim for tighter glucose control in patients with T2DM and CKD without exaggerating their risk for all cause and cardiovascular mortality[9].

GLUCOSE LOWERING AGENTS AND RENAL PROTECTION

Older glucose lowering medications

No large clinical studies have specifically examined the renal protective effects of insulin, sulphoylureas or metformin apart from the possible effects derived from improved glucose control.

Thiazolidinediones

The thiazolidinediones (TZDs) activate the peroxisome proliferator-activated receptor-Gamma and improve glucose control by improving insulin sensitivity. This class of medication has also been reported to have anti-inflammatory and antithrombotic effects but they also cause weight gain and fluid retention in some patients. Animal studies have shown that TZDs reduce albumin excretion, prevent glomerulosclerosis, tubulo-interstitial fibrosis and maintain podocyte structure and function. Although a meta-analysis of 15 studies involving 2860 patients has shown that TZDs can significantly decrease urinary albumin excretion it should be noted that not all studies have found a favourable effect of TZDs on urinary albumin excretion[48]. For example, in the Diabetes Reduction Assessment With Ramipril and Rosiglitazone Medication trial, rosiglitazone reduced a combined renal endpoint by 20% in people with prediabetes (HR = 0.80, 95%CI: 0.68-0.93, P = 0.005) over the 4 years of the trial. Prevention of diabetes was also independently associated with prevention of the renal endpoint of the trial (P < 0.001). In contrast, in the bypass angioplasty revascularisation investigation 2 Diabetes trial, participants who were treated with insulin sensitising medications (the majority taking TZDs in combination with

metformin), as compared to those treated with insulin-provision therapy (insulin plus sulphonylureas), had greater progression of urinary albumin excretion despite having lower HbA1c values. Rates of decline in estimated GFR were reported to be similar in both treatment groups over 5 years[49].

Incretins

There is an emerging signal to suggest that the incretin based therapies for type 2 diabetes such as the dipeptidyl peptidase-4 (DPP-4) inhibitors and the glucagon-like peptide-1 (GLP-1) receptors may have specific renal protective properties. DPP-4 inhibitors and GLP-1 receptor agonists target the "incretin effect" which involves the modulation of peptide hormones that normally regulate glucose levels when nutrients are given orally. The incretins are a family of gut hormones that lower blood glucose levels *via* the so called "incretin effect". This phenomenon accounts for the two-to-three fold increase in plasma insulin concentrations observed after the oral ingestion compared to the intravenous administration of an equivalent glucose load. The principal incretin peptide hormone that has been targeted for thermotherapy is GLP-1 secreted by intestinal L cells[50].

Normally GLP-1 has a very short half-life and is quickly degraded by the DPP-4 enzyme. Two pharmacological approaches have been taken to target the "incretin-system" to develop new glucose lowering medications. One approach has been to develop GLP-1 receptor analogues that are resistant to degradation by the DPP-4 enzyme, hence enhancing their half-life. The other approach has been to develop inhibitors of the DPP-4 enzyme, with the aim of increasing plasma levels of native GLP-1 by inhibiting its degradation[51].

GLP-1 and DPP-4 inhibitors have been shown to inhibit the sodium-hydrogen ion exchanger in the proximal tubule which increases sodium excretion and triggers tubulo-glomerular feedback to constrict the afferent glomerular arteriole and hence reduce intra-glomerular pressure as described previously. The potential renal protective effects of these medications likely involves deceases in oxidative stress, inflammation and glomerulosclerosis[52].

Experimental studies involving GLP-1 receptor knockout mice with diabetes have shown that they exhibit higher urinary albumin levels and more advanced mesangial expansion than wild-type mice with diabetes, despite comparable levels of hyperglycaemia. Increased glomerular superoxide and upregulated renal NOX were also seen in the GLP-1 receptor knockout mice. Furthermore, treatment with the GLP-1 receptor agonist liraglutide suppressed the progression of nephropathy in mice with diabetes, as demonstrated by reduced albuminuria and mesangial expansion, decreased levels of glomerular superoxide and renal NOX. These results suggest that GLP-1 normally plays a crucial role to maintain kidney health by protecting against increased renal oxidative stress induced by chronic hyperglycemia[53].

Information regarding the microvascular benefits of the GLP-1 receptor agonist Liraglutide has also just been released. The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome (LEADER) trial examined the effects of a daily injection of liraglutide *vs* placebo in 9340 high risk vascular patients over a median follow-up of 3.8 years. Approximately, 23% of the trial participants had an eGFR < 60 mL/min per 1.73 m^2 whist 21% had microalbuminuria and 6% had macroalbuminuria. The use of liraglutide was associated with a reduction in the primary end point of the trial which was a composite CV end point of death due to CV disease, non-fatal myocardial infarction and non-fatal stroke (HR = 0.87, 95%CI: 0.78-0.97, P = 0.01). There was also a 22% significant reduction in the time to the first primary renal endpoint of the trial, defined as a composite of the development of macroalbuminuria, doubling of serum creatinine and eGFR < 45 mL/min per 1.73 m^2, the need for renal replacement therapy and death from renal causes (HR = 0.78, 95%CI: 0.67-0.92, P = 0.03). The renal benefit of liraglutide was mainly derived from a 26% significant reduction in new onset macroalbuminuria (HR = 0.74, 95%CI: 0.60-0.91) without any significant changes in eGFR. Lower, but non-significant rates of doubling of serum creatinine and the need for the initiation of renal replacement therapy were also seen in liraglutide treated patients (Figure 1). Furthermore, most of the CV benefits observed with liraglutide were seen in patients with eGFR levels < 60 mL/min per 1.73 m^2 (Figure 2A)[54].

The mechanisms whereby liraglutide reduces progression to macroalbuminuria remain unknown but may be in part related to improved metabolic control and modulation of inflammatory pathways. In the LEADER study, the use of liraglutide *vs* placebo was associated with decreases in HbA1c, weight and systolic blood pressure of 0.4%, 2.3 kg and 1.2 mmHg, respectively. Of relevance to the reno-protective effects of the SGLT-2 inhibitors discussed below, GLP-1 receptor agonists do not appear to have any direct effect on renal haemodynamics. A recent study has shown that short term infusion of the GLP-1 receptor agonist exenatide had no effect on directly measured GFR (inulin clearance) and renal blood flow (para-aminohippurate clearance), but did induce a natriuresis[55].

After this manuscript was submitted for review the results of the Trial to Evaluate Cardiovascular and other long-term outcomes with semaglutide (SUSTAIN-6) have been released. This trial showed that a once weekly injection of semaglutide significantly reduced CV endpoints in high risk vascular patients with type 2 diabetes. In a similar fashion to liraglutide it also reduces progression to macroalbuminuria (HR = 0.54, 95%CI: 0.37-0.77, P < 0.001). It should also be noted that currently, liragludite and semaglutide have only been shown to reduce progression to macroalbuminuria and that their ability to reduce progression to ESKD remains to be proven[54,56].

The results of large clinical studies investigating the potential reno-protective effects of DPP-4 inhibitors have not been published to date. However, the renal outcomes for a pooled analysis of clinical trial data involving 3505

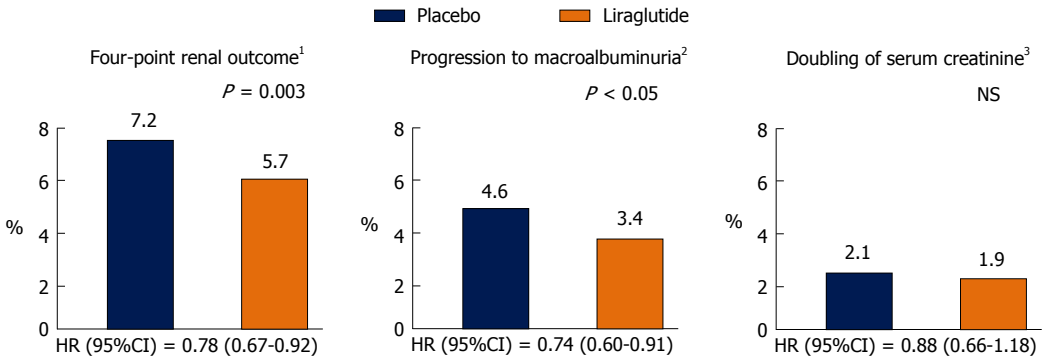

Figure 1 Renal outcomes in the trial Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome. [1]Four-point renal outcome = progression to macroalbuminuria, doubling of serum creatinine, initiation of RRT or death from renal disease; [2]Macroalbuminuria = albumin to creatinine ratio > 30 mg/mmol; [3]Plus eGFR < 45 mL/min per 1.73 m^2. eGFR: Estimated glomerular filtration rate; HR: Hazard ratio; NS: Non statistically significant; RRT: Renal replacement therapy.

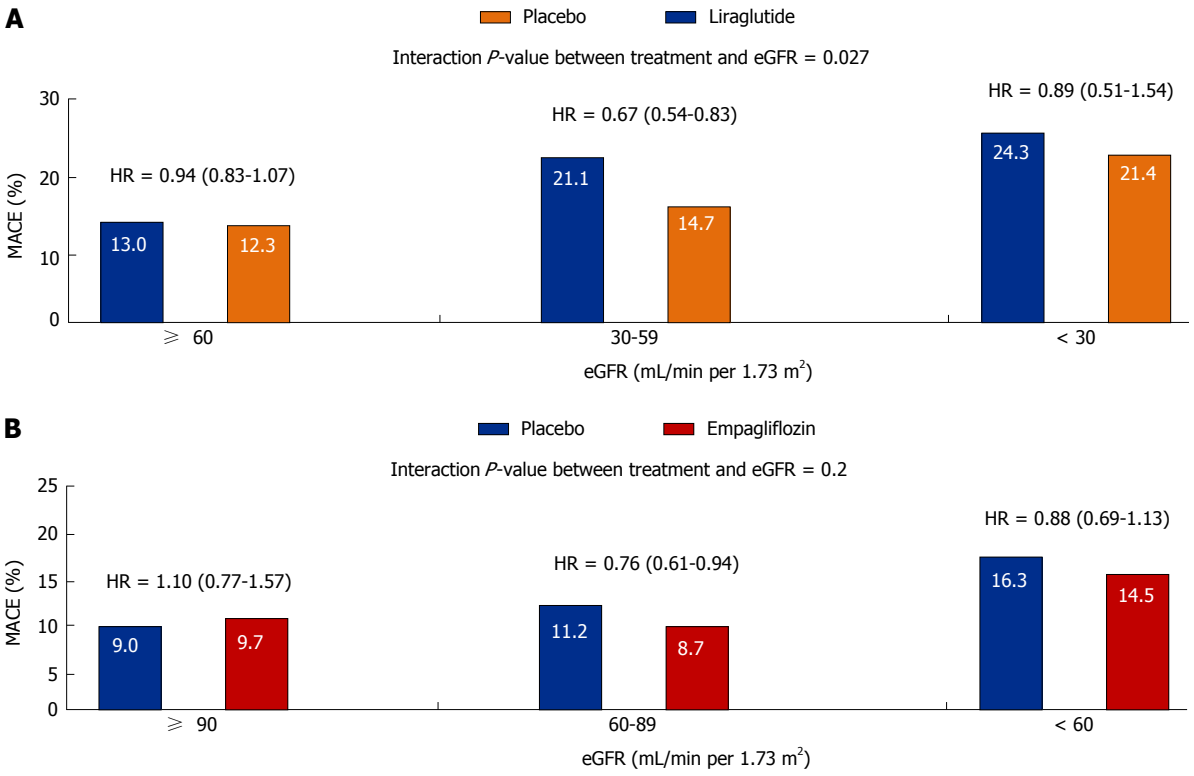

Figure 2 Cardiovascular outcomes in the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome (A) and cardiovascular safety trial of empagliflozin (B) studies according to estimated glomerular filtration rate. MACE: Major adverse cardiovascular event; eGFR: Estimated glomerular filtration rate; HR: Hazard ratio.

participants treated with the DPP-4 inhibitor linagliptin and 1961 placebo treated participants with a median treatment exposure of 171 d (range, 1-531) for linagliptin and 172 d (range, 1-531) for placebo have been published. The primary renal outcome was defined as first occurrence during the study of 6 predefined end points: New onset of microalbuminuria, new onset of macroalbuminuria, reduction in kidney function (serum creatinine increase to ⩾ 250 μmol/L), halving of estimated glomerular filtration rate (loss of baseline eGFR > 50%), acute renal failure (ascertained from diagnostic codes), or death from any cause. The primary composite outcome occurred in 448 (12.8%) and 306 (15.6%) participants in the linagliptin and placebo groups, respectively. Linagliptin treatment significantly reduced the hazard of kidney disease events by 16% compared with placebo (HR = 0.84; 95%CI: 0.72-0.97, P = 0.02). A sensitivity analysis showed that adjustment for kidney function at baseline did not influence the association between reduced renal risk and linagliptin treatment (HR = 0.83; 95%CI: 0.72-0.97, P = 0.02). Moreover, the observed risk reduction for kidney disease end points with linagliptin was consistent across examined subgroups, including those using renin-angiotensin system (RAS) inhibitors.

Overall medications that target the incretin effect are

generally well tolerated. The DPP-4 inhibitors are virtually free from side-effects and usually can be used at any level of renal function with an appropriate dose reduction. It should however, be noted that Linagliptin is not renally excreted and therefore does require a dose reduction at lower GFR levels. The side-effects of GLP-1 receptor agonists are mainly related to the gastrointestinal tract and include nausea, vomiting and gallstone disease. Concerns that medications targeting the incretin effect may promote the development of pancreatitis and pancreatic cancer have been raised but have not been supported by the results of large randomised clinical trials. A possible link between the use of liraglutide and semiglutide and the promotion of diabetic retinopathy has also been raised. The significance of these findings remains to be fully established. An important point practice point to highlight for patients with CKD is that the GLP-1 receptor agonists are currently not recommended for used in patients with an eGFR < 30 mL/min per 1.73 m^2.

SGLT-2 inhibitors

The SGLT2 receptor mediates high-capacity glucose uptake in the early proximal tubule, and SGLT2 inhibitors, *via* their ability to promote glycosuria, have been developed as glucose lowering medications. There is an emerging body of evidence suggesting that this class of medication may have an important role in reducing intraglomerular pressure which is then in part translated into a reno-protective effect.

In hyperfiltering patients with T1DM, the SGLT-2 inhibitor empagliflozin has been shown to reduce GFR and resolve hyperfiltration in short term studies by counteracting the tubulo-glomerular feedback mechanism. Specifically, SGLT-2 inhibitors constrict the afferent glomerular arteriole in response to an increase in tubular sodium sensed by the macular densa. This increase in tubular sodium results from less sodium being reabsorbed in the proximal tubule due to inhibition of sodium-glucose transport in the proximal tubule.

In a recent euglycaemic clamp study involving 13 normofiltering and 27 hyperfiltering T1DM subjects, eight weeks of treatment with empagliflozin resulted in a reduction in GFR of 33 mL/min per 1.73 m^2 in the hyperfiltration group without any change in GFR in the normofiltering group[42]. A similar degree of GFR reduction associated with RAS inhibitors was observed in previous studies of hyperfiltration in young patients with uncomplicated T1DM[57]. Apart from the above, SGLT-2 inhibitors may possibly have additional renal protective effects as they improve glycaemic control, lower blood pressure and promote weight loss[58].

In the landmark CV safety trial of empagliflozin (EMPA-REG), 7020 patients T2DM patients at high risk for CV events who received empagliflozin (with no differences seen for 10 or 25 mg doses) had reduced rates of CV and all-cause mortality when compared with placebo when studied for 3.1 years. Reductions in death from CV causes (HR = 0.62, 95%CI: 0.49-0.77, $P < 0.001$), hospitalization for heart failure (HR = 0.65, 95%CI: 0.5-0.85, $P = 0.002$) and death from any cause (HR = 0.68, 95%CI: 0.57-0.82, $P < 0.001$) where seen in empagliflozin compared with placebo treated participants, respectively[45].

Furthermore, a recent follow-up study from the EMPA-REG OUTCOME study has shown that empagliflozin reduces clinically important renal outcomes. All patients in the study had an eGFR > 30 mL/min per 1.73 m^2 and approximately 25% had eGFR < 60 mL/min per 1.73 m^2, 11% had macroalbuminuria, 29% had microalbuminuria and 80% were on RAS blockers. The primary renal end point of the trial was a four point composite of new onset or worsening of nephropathy (progression to macroalbuminuria, doubling of serum creatinine level associated with an eGFR < 45 mL/min per 1.73 m^2, initiation of renal replacement therapy and death from renal disease). This endpoint occurred in 18.8% of placebo and 12.7% of empaglifozin treated patients which resulted in a risk reduction of 39% in patients that received empaglifozin (HR = 0.61, 95%CI: 0.53-0.7, $P < 0.001$). In a similar fashion to what seen for CV outcomes in the EMPA-REG study, there were no differences in the renal outcomes for patients receiving the 10 or 25 mg doses of empagliflozin. Importantly, the impact of empagliflozin on the primary CV (Figure 2B) and renal end-points was not diminished in patients with CKD compared to those without CKD[46].

The main driver for a reduction in the primary renal endpoint in empaglifozin treated patients was a slowing in progression to macroalbuminuria, 16.2% *vs* 11.2% in placebo and empaglifozin treated patients, respectively (HR = 0.62, 95%CI: 0.54-0.72, $P < 0.001$). Several sensitivity analyses were therefore performed to test the strength of the relationship between empagliflozin treatment and the reduction in clinically meaningful renal endpoints. This analysis showed a consistent empagliflozin effect on indices of renal health as summarised in Figure 3. Of note, empaglifozin *vs* placebo treatment resulted in a 46% risk reduction in the traditional clinically meaningful composite endpoint of doubling of serum creatinine levels (plus in this study achieving an eGFR < 45 mL/min per 1.73 m^2), initiation of renal replacement therapy and death from renal disease (HR = 0.54, 95%CI: 0.40-0.75, $P < 0.001$).

In empagliflozin treated patients there was an initial drop in eGFR of approximately 4 mL/min per 1.73 m^2 but at the end of the 5 years of the trial eGFR values were 4.7 mL/min per 1.73 m^2 higher in patients that received empagliflozin. This difference in eGFR values appears to be accounted for by a progressive drop in eGFR values in the placebo treated patients, which could be related to the expected decline in GFR that is associated with aging, and a stabilisation of GFR values in empaglifloszin treated patients after the initial drop in eGFR described above. This initial drop in eGFR and rapid increase in eGFR values that was seen after discontinuation of empagliflozin are consistent with a direct effect of the medication on renal haemodynamics, as discussed

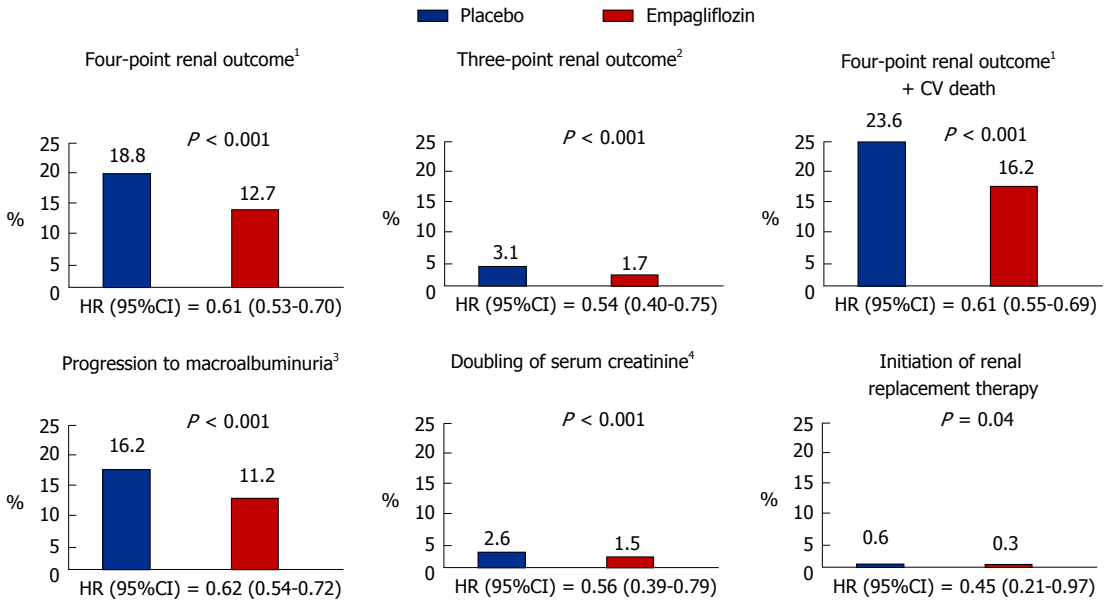

Figure 3 Renal outcomes in the cardiovascular safety trial of empagliflozin study. [1]Four-point renal outcome = progression to macroalbuminuria plus three-point renal outcome; [2]Three-point renal outcome = doubling of serum creatinine, initiation of RRT or death from renal disease; [3]Macroalbuminuria = albumin to creatinine ratio > 30 mg/mmol; [4]Plus eGFR < 45 mL/min per 1.73 m^2. HR: Hazard ratio; eGFR: Estimated glomerular filtration rate; CV: Cardiovascular; RRT: Renal replacement therapy.

above.

The above findings also suggests that a reduction in intraglomerular pressure ultimately results in a preservation of eGFR and is the main mechanism responsible for lower rates of progression to clinically meaningful renal end points for empagliflozin treated patients in EMPA-REG. The rapid divergence in the rates of development of the primary renal endpoint, within 3 mo, in the EMPA-REG study over time also suggests that direct haemodynamic effects rather than improvements in metabolic parameters were primarily responsible for the improvement in renal outcomes seen in the trial. A recent study has also shown that empagliflozin can reduce albuminuria in micro- and macroalbuminuric patients and that this effect is mainly independent of the metabolic and known systemic haemodynamic effects of SGLT-2 inhibitors[59]. The above finding also supports a direct renal effect of SGLT-2 inhibitors. However, contributions from decreases in HbA1c, weight, blood pressure and uric acid levels that are observed in SGLT-2 inhibitor treated patients should also be considered. Furthermore, it has recently been suggested that the use of empagliflozin results in a shift in renal and cardiac fuel metabolism from fat and glucose oxidation to ketone bodies and that this metabolic substrate shift improves the function of these organs[60,61].

Potential side-effects or concerns related to the use of SGLT-2 inhibitors include increased rates of urinary tract infections, genital tract infections, postural hypotension, diabetic ketoacidosis, acute kidney injury and possible increased rates of fractures[58,62-64]. Furthermore, the main disadvantage of the mode of action of the SGLT-2 inhibitors is that their effectiveness for lowering blood glucose levels is dependent on renal function. Hence they are not recommended as glycaemic management agents in patients with significantly impaired renal function.

CONCLUSION

In summary, the HbA1c threshold for the development of kidney dysfunction remains to be clearly defined but is possibly around 6.5%. Achieving this threshold should be balanced against the increasing appreciation that glucose targets for the prevention of diabetes related complications need be individualised for each patient. Tight glucose control has clearly been shown to reduce the incidence of micro- or macroalbuminuria. However, it is only in very recent times that some evidence has emerged to suggest that intensive glucose control can slow GFR loss and possibly progression to ESKD. The concept of "metabolic memory" is again highlighted in the DCCT/ECIC study of changes in GFR. This type of study emphasises the importance of early intensive glucose control in delaying the development of subsequent diabetes related complications.

Furthermore, recent large randomised clinical trials have shown that the glucose lowering medications liraglutide, semaglutide and empagliflozin have renal protective effects. The mechanisms whereby there medications improve renal outcomes remain to be fully elucidated but are almost certainly over and above those expected from their glucose lowering effects alone with a decrease in intra-glomerular pressure appearing to be a likely mechanism that links the use of empagliflozin with lower rates of progression to ESKD.

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REVIEW

Effect of medicinal mushrooms on blood cells under conditions of diabetes mellitus

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Abstract

Diabetes mellitus (DM) is the third most common non-infectious disease leading to early disability and high mortality. Moreover, the number of patients is growing every year. The main symptom of DM is hyperglycemia. Increased levels of blood glucose activate polyol, hexosamine, and protein kinase metabolic pathways cause the intensification of non-enzymatic glycosylation and nitration of macromolecules. This, in turn, leads to the development of oxidative and nitrative stresses and secondary complications, such as different kinds of micro- and macroangiopathies. Metabolic disorders caused by insulin deficiency in diabetes significantly impede the functioning of a homeostasis system, which change the physical, biochemical, morphological, and functional properties of blood cells. As a result, the oxygen-transport function of red blood cells (RBCs), rheological properties of the blood, and functions of immunocompetent cells as well as the process of apoptosis are primarily affected. Modern pharmacotherapy focuses on the search for new preparations that aim to decrease blood glucose levels. Undesirable side effects and adverse reactions caused by synthetic medicines led to the search and investigation of new preparations of natural origin. Medicinal mushrooms play an important role among such new preparations. They are a source of a large number of high- and low-molecular compounds with pronounced biological effects. Our investigations show pronounced hypoglycemic and anti-anemic action of submerged cultivated mycelium powder of medicinal mushrooms *Agaricus brasiliensis* (*A. brasiliensis*) and *Ganoderma lucidum* (*G. lucidum*) on streptozotocin-induced DM in rats. Also, we showed that mycelium powders have membrane protective properties as evidenced by the redistribution of RBC populations towards the growth of full functional cell numbers. Normalization of parameters of leukocyte formula and suppression of apoptosis of white blood cells in diabetic

rats treated with *A. brasiliensis* and *G. lucidum* mycelia indicates pronounced positive effects of these strains of mushrooms. Thus, the use of medicinal mushrooms for treatment of DM and in prevention development of its secondary complications might be a new effective approach of this disease's cure. This article is aimed at summarizing and analyzing the literature data and basic achievements concerning DM type 1 treatment using medicinal mushrooms and showing the results obtained in our research.

Key words: Diabetes mellitus; Streptozotocin; *Agaricus brasiliensis*; *Ganoderma lucidum*; Leukocytes; Red blood cells

Core tip: Diabetes mellitus (DM) is the third most common non-infectious disease leading to early disability and high mortality. The main symptom of DM is hyperglycemia. Metabolic disorders caused by insulin deficiency in diabetes significantly impede the functioning of a homeostasis system. Medicinal mushrooms play an important role among new preparations that aim to decrease blood glucose levels. This investigations show pronounced hypoglycemic, anti-anemic, membrane protective, apoptosis suppressive effects of *Agaricus brasiliensis* and *Ganoderma lucidum* on streptozotocin-induced DM in rats. Thus, medicinal mushrooms might be new effective approach for treatment and prevention of DM.

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INTRODUCTION

According to the World Health Organization, diabetes mellitus (DM) is a chronic disease caused by the diminished secretion of insulin, the hormone produced by the pancreas and regulating the level of glucose in the blood, or by increasing resistance of the body's cells to this hormone[1].

According to International Diabetes Federation data, in 2015 there was 415 million of people suffering from diabetes worldwide. Almost half of them are men aged 40 to 59 (7% more than women). If the tendency for diabetes continues to grow, the number of people suffering from this disease will reach 642 million people in 2040. It should also be noted that about 80% of diabetics live in low- and middle-income countries[2].

There are three main types of DM: Type 1 diabetes (T1D) (previously known as insulin-dependent or juvenile-onset; accounting for 3%-10% of cases); type 2 diabetes (T2D) (previously known as non-insulin-dependent or adult-onset; accounting for 85%-90% of cases); and gestational diabetes (hyperglycemia occurring during pregnancy and usually resolving itself after delivery; accounting for 2%-5% of cases)[3]. Also, there are other specific types of diabetes with unknown etiology[4].

Although the percentage incidence of T1D is relatively small (*e.g.*, compared with T2D), it is common in children and adolescence. This sets a particular danger associated with early disability and increases the rates of lethal cases[5]. T1D results from a selective destruction of pancreatic β-cells caused by cytotoxic lymphocytes, T-helpers and autoantibodies. The progressive destruction of β-cells results in insulin deficiency, which leads to dyscrasia and diabetes[6]. The etiology of insulin-dependent diabetes has not been fully examined. T1D is considered to be caused by an interaction between genetic and environmental factors among which include viruses and genetic mutations[7]. It is observed that some viruses can trigger autoimmune processes, promote them, or assume both roles simultaneously[4]. Some scientists consider that one or more viruses can also cause T1D among individuals with genetic susceptibility. The scientists presume that in the presence of infection a virus protein similar to β-cells protein is produced. T-cells and antibodies to foreign proteins attack the native proteins of pancreatic β-cells[4]. A range of viruses exists which share these properties, for instance, intestinal Coxsackie viruses. Accordingly, autoimmune processes contribute to the destruction of the islets of Langerhans that leads to diabetes. Yet, at this stage, genetic damages play a main role in causing autoimmune processes that result in T1D. Scientific researches reveal that insulin-dependent DM is inflicted not by a violation of any particular section of the genome, but by complex damage. Genes in Human Leucocyte Antigens-locus or genes of the major histocompatibility complex are responsible for T1D. The development of T1D is associated with genetic determination. Antigens of the major histocompatibility complex account for the susceptibility to disease with various immune responses[8]. Genetic investigations indicate that type 1 DM is strongly associated with at least 18 loci[9]. However, the precise mechanisms that lead to autoimmune destruction of β-cells in T1D remain poorly understood. According to modern concepts of the pathogenesis of the disease, the destruction of β-cells can occur as a result of various pathological processes such as necrosis and/or apoptosis[10]. Cytotoxic T lymphocytes are considered to be a main factor that induces the destruction of β-cells; their production is a crucial factor for this pathology. Most autoreactive T-cells of Langerhans islets produce cytokines that are characteristic of T × 1-lymphocytes, particularly, a tumor necrosis factor alpha, interferon gamma, IL-2. The latter is known to trigger a cellular immune response in which cytotoxic effectors T lymphocytes attack β-cells and induce antigen specific T-dependent immune lysis. The development of type 1 DM depends not only on the presence of autoreactive T-lymphocytes, but also

on control mechanisms that ensure the maintenance of immune tolerance or inhibition of peripheral autoimmune processes[11].

The mechanism of β-cells destruction is complicated, slow and gradual, and the consecution of pathogenetic processes is not fully elucidated. However, when insulin deficiency becomes close to absolute (in which approximately 85% of β-cells are destroyed), it induces severe metabolic disorders and a clinical stage of disease occurs[12].

Thus, DM is still one of the main problems of the 21st century. The search of new, safe and available for most of people approaches in its treatment is relevant trend of investigation.

STRUCTURAL AND FUNCTIONAL DISORDERS OF BLOOD CELLS UNDER DM

Morphological and functional changes of red blood cells under conditions of T1D

Red blood cells (RBCs) are the largest blood fraction, with unique cells lacking nuclei in the shape of biconcave disks[13]. The main function of the cells is to transport oxygen by means of hemoglobin from the lungs to all organs and tissues and then transport CO_2 in the opposite direction. In adults, RBCs develop in the bone marrow and circulate for about 120 d in the bloodstream before they are eliminated by Kupffer's cells of the liver and spleen. This process is slow and multistage and is controlled by hormones (*e.g.*, erythropoietin) and cytokines. RBCs enter in the bloodstream at the stage of the reticulocyte. Reticulocytes do not have nuclei, however, they have a residual protein synthesis apparatus. They account for about 1%-2% of circulating RBCs and are transformed into mature cells within 24-48 h[14].

Metabolic disorders caused by insulin deficiency under DM considerably change physical, biochemical, morphological, and functional properties of RBCs. The membrane as a single structural element of these cells plays an important role in the maintenance of the cell's stable form and provides its functional properties. Under DM, profound changes in the RBC membrane structure, which affect physicochemical properties of cells, are brought about. Thus, lipid exchange is disturbed, and the molecular architectonic of the red cell membrane lipid bilayer is changed[15]. It has been revealed that the cholesterol level rises due to a nonspecific exchange mechanism with plasma cholesterol, which is higher in diabetics[16]. The level of phospholipids with increased content of unsaturated fatty acids in RBC membranes also rises[17]. These changes lead to a decrease in membrane fluidity, which corresponds to enhanced lipid-lipid interactions, decreased protein-lipid interactions, and increased protein-water interactions resulting in displacement of proteins towards the membrane surface. Moreover, the cholesterol level is considered to affect the transport of substances *via* the membrane[16].

Under DM, the membrane's total protein content (glycoproteins in particular) is shown to decrease, whereas the activity of sialidases increases[18]. Increased activity of these enzymes, in turn, causes a decrease in sialic acids on RBC surfaces. As a result, a superficial negative charge of the cells changes, aggregation properties increase, and their deformability decreases thus increasing blood viscosity, complicating its flow through microcirculation and acting as a precursor of diabetic complications.

Due to hyperglycemia, the processes of non-enzymatic glycosylation of red cell membranes and cytosol proteins are activated. An increase in glycosylated hemoglobin (HbA1c) affects the efficiency of the oxygen-transport function of RBCs. HbA1c has an enhanced affinity to O_2, which complicates its return to cells in microcirculation[19]. That, in turn, promotes the development of tissue hypoxia. There is an enhanced level of fetal hemoglobin (HbF) in RBCs under the condition of diabetes. Such changes are compensatory, providing a better supply of oxygen to tissues under DM, as HbF is able to bind oxygen with greater affinity and return it at much less partial pressure[20].

In recent years, the role of RBCs and hemoglobin has been intensively discussed both as inhibitors and activators of nitric oxide (NO)-dependent signaling in NO metabolism[21]. Previously, it had been believed that RBCs fulfill solely the role of nitric oxide scavengers and its depot. However, functionally active isoforms of NO-synthase (an enzyme which produced nitric oxide) have been found in RBCs[22-24].

It is known that the NO produced in RBCs is involved in cell deformation processes providing their passage through microvessels and function performance[25]. Apart from that, nitrogen monoxide may diffuse from red cells to plasma inhibiting platelet activation and aggregation, as well as adhesion and migration of leukocytes[26]. In addition to NO formation, RBCs occupy a key role in nitric oxide pool support in the bloodstream. "Unused" NO diffuses from plasma to cells where it is turned into stable products by means of hemoglobin. On the one hand, hemoglobin takes part in the formation of stable NO metabolites, and on the other - it has nitrite reductase activity that underlies RBC-dependent vasodilatation[27]. This reaction plays a significant role under hypoxia conditions that develops during DM. Moreover, hemoglobin is involved in the deposition of NO in nitrosyl hemoglobin and S-nitrosohemoglobin forms. Mutual transformations of NO-hemoglobin derivatives under certain conditions lead to the release of this vasodilatator[28,29].

Under DM, several factors contribute to a decrease in NO bioavailability: Reduced production by NO-synthase[28]; reduced availability of substrate of NO-synthase (*i.e.*, L-arginine)[30,31]; its inactivation by reactive oxygen species (ROS)[28]. Reduced NO bioavailability causes disturbances in RBC deformability[24] and, as a result, complicates their microcirculation, thus leading to the development of vessel complications and hypoxia. HbA1c

(in the form of nitrosothiols) is known to bind NO rather tightly, which adversely affects NO diffusion into muscle cells and, respectively, their relaxation[32].

Changes of RBCs in patients with diabetes cannot be considered without taking into account the specificity of erythropoiesis during this disease. Erythropoiesis is increased in hyperglycemia conditions and accompanied by a growing number of reticulocytes in the bloodstream. The erythropoiesis stimulation can be caused by an increase in erythropoietin synthesis in response to tissue hypoxia (kidney tissues in particular). The lifespan of RBCs is shortened by 13% against the background of elevated erythropoiesis under DM[15,33].

Under hyperglycemia, autoxidation of glucose occurs, which is considered to be the major mechanism for free radical formation in RBCs[34]. In its enediol form, glucose with the help of transition metals is oxidized to an enediol-anion radical, which, in turn, is transformed into reactive ketoaldehydes and superoxide anion radical. O_2^-• is involved in the formation of other radicals, hydroxyl radical (•OH) and peroxynitrite in particular. Hyperglycemia promotes lipid peroxidation through a superoxide-dependent pathway, which also provides the formation of free radicals[35]. Interaction between glucose and protein amino acid residues leads to formation of Amadori products and advanced glycation end-products (AGEs). Interacting with corresponding receptors, AGEs inactivate enzymes changing their structure and function[36], thus promoting free-radical formation[37] and blocking the antiproliferation effect of NO[38]. In addition, NADH oxidase enzymes and NO-synthase isoforms, found recently in RBCs, contribute to the formation of endogenous oxidants and development of oxidative stress[39].

Changes effected by oxidative stress involve plasma proteins, membranes, and lipids. Hemoglobin is gradually oxidized to methemoglobin through long-lasting exposure to a high concentration of oxygen. Hemoglobin oxidation reduces the amount of oxygen supplied to tissues and leads to the formation of disulfide bridges between globin chains. Eventually, Heinz bodies are produced, and hemoglobin is precipitated followed by macrophage phagocytosis or RBC lysis[40]. Under the interaction between ROS and other RBC proteins, amino acids of protein molecule side chains are oxidized; protein-protein linkages are formed; protein chains are oxidized with further fragmentation and a lot of protein oxidation products are produced[41]. Such modifications can occur both in structural proteins of the cytoskeleton and membrane and in enzymes causing disturbances in their functionality.

Membrane lipids also undergo oxidation, especially polyunsaturated fatty acids, which are in abundance in the RBCs membrane. As a result, elasticity and fluidity are affected, as well as deformability and shape of red cells, which in turn influence the functional state of RBCs[42]. Since lipids are not synthesized in RBCs and the exchange is restricted between lipids and plasma[43], the damage is accumulated and eventually leads to either premature sequestration and RBCs elimination from the bloodstream or their lysis.

Morphological and functional changes of immune competent blood cells under conditions of DM type 1

White blood cells are the cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders. The main function of leukocytes is to provide the specific and the non-specific immune defenses of the body through the cells with effector and immunoregulatory activity. Diabetes is often accompanied by infectious and inflammatory processes that occur with relapses and are difficult to treat. Changes in the count of leukocytes and their ratio may be a cause of susceptibility to infectious and inflammatory processes in patients with DM. It has been shown that the development of diabetes is accompanied by leukocytosis[44] against a background of a reduction in the number of lymphocytes. Such changes may lead to impairment in functional properties of the immune system which is an additional risk factor for disability in patients with diabetes or even death[45]. In T1D a disturbance of cellular immune response occurs, which is accompanied by changes in the activity of different subpopulations of cells: By exhaustion of T lymphocytes, reduction of T suppressors' activity, an increase in activated T lymphocytes and T helpers' quantity and by violation of IL-2 production by T lymphocytes. Also, the functional activity of peripheral blood monocytes is reduced, although their number in diabetic patients may be increased. In leukocytes the inhibition of key enzymes of glucose anaerobic oxidation and decrease of intracellular ATP reserves occurs. The development of hyperglycemia in DM leads to a disruption of polymorphonuclear leukocyte functioning, resulting in reduced intensity of a "respiratory explosion" and their phagocytic activity during the response to bacterial infection[46].

Other important functional changes that cause angiopathy are: Increased permeability of the vascular wall, hemodynamic disorders, changes in blood viscosity, and a violation of adhesive, aggregation and migration ability of WBCs, which are dependent on their morphological and functional states. The development of an immune response occurs as a result of an interaction between leukocytes and antigens, soluble regulatory molecules and with other leukocytes. To initiate an inflammatory response to a bacterial infection, leukocytes have to migrate from the blood into the affected tissues, in relation to which they have positive chemotaxis. Peripheral blood leukocytes, in particular, neutrophilic granulocytes and lymphocytes, have a leading role in the pathogenesis of diabetic complications. The mechanism causing damage to the vascular endothelium by leukocytes is not completely elucidated, but it is known that the interaction between leukocytes and endothelial cells provides adhesive molecules, most of which are part of the membrane receptors of leukocytes and, according to chemical structure, are sialoglycoproteins. The increase in the number of exposed surface cells (sialoglycoconjugates) correlates with the damage of many cell types[47,48]. The

potential significance of carbohydrate-specific interactions in the regulation of cellular functions is very important because an abundance of glycoligand structures expressed on the cell surface can be modified under hyperglycemic conditions in DM. Content redistribution and structural changes in carbohydrate determinants of glycoproteins on the plasma membrane of leukocytes under type 1 DM may contribute to an increased adhesion of leukocytes to the vascular endothelium and to the disruption of their phagocytic functions. These interactions could be the reasons for complications in clinical treatment of the disease.

Many pathological states, for example, diabetes, are accompanied by changes in sialic acid expression[49]. Under diabetes, leukocytes demonstrate increased adhesive ability to the vascular endothelium. This pathogenetic mechanism is mediated by an elevated level of cell adhesion molecules on the surface of endothelial cells and leukocytes and causes abnormal leukocyte-endothelial cell adhesion. Increasing membrane rigidity and reducing the ability of WBCs to deform may damage the capillaries. And next to the small diameter of the lumen of blood vessels, increased adhesion of leukocytes to the endothelial wall leads to their capture in capillaries (leykostaz) and to increased vascular occlusion, which is an important point in the development of diabetic microangiopathies[50].

The result of hyperglycemia in the cell is the excessive accumulation of products of nonenzymatic glycosylation in cells, and also violates many biochemical and physiological parameters causing cell depletion of energy resources and antioxidants. Accumulation of AGEs either inside or outside the cell perturbs the functions of molecules and the cell on the whole[51]. Some proteins that regulate the transcription of certain genes can participate in the process of glycosylation. The molecules of AGEs, when on the cell surface, can affect signaling and interaction between intercellular space and cells, thus disrupting cell functions. The disruption of the electron transport chain and excessive generating of ROS occur in the mitochondria. Overproduction of mitochondrial superoxide during hyperglycemia is a primary initiating mechanism that activates pathways of diabetes' vascular tissue damage, leading to cellular redox imbalance and oxidative stress[52,53]. It is known that inflammatory processes are characterized by the activation of immunocompetent cells with the corresponding activation of inducible NO-synthase, and a local increase of concentration of NO hundreds of times above the norm, depending on the cell type[54]. Accordingly, under conditions of oxidative stress and high content of O_2^{-}• elevated concentration of NO leads to the reaction between them and induces increased concentration of peroxynitrite. In turn, peroxynitrite might affect different molecules causing their modification.

Protein tyrosine nitration causes various changes in protein structure and function and catalytic activity of enzymes. Protein tyrosine nitration, including a covalent modification of the phenolic ring, may also affect tyrosine phosphorylation/dephosphorylation. For example, peroxynitrite affects the insulin signal transduction through tyrosine nitration[55] and contributes to pathological complications of a whole range of diseases. Thus, development of oxidative-nitrative stress is accompanied by protein nitration with resultant changes in cell signaling, DNA single-strand breakage and base modification, and the formation of products which exhibit potent proapoptotic effects[56]. In addition, overproduction of ROS by high glucose compromises the antioxidant defense mechanisms, such as reduced levels of mitochondrial-specific manganese superoxide dismutase, and further aggravates oxidative stress[57]. Under diabetic and high-blood glucose conditions, excessive ROS might lead to damage of the structure and function of mitochondria, therefore prompting the release of apoptosis-inducing factors such as cytochrome C[58]. A high occurrence of apoptotic lymphocytes has been shown in diabetic patients, resulting in reduced numbers of circulating lymphocytes in these patients. A number of studies suggest that dysregulated apoptotic immune cell death may play a role in contributing to the immune dysfunction[59]. The key apoptotic proteins regulators in the mitochondrial pathway are the Bcl-2 superfamily proteins. DNA damage or intra/extra-cellular stress initiates signaling cascades in the cytoplasm, eventually leading to the phosphorylation of p53 which, in turn, increases Bax concentration while lowering Bcl-2[60]. Bax expression promotes apoptosis, whereas Bcl-2 expression protects cells against oxidative stress and inhibits apoptosis[61]. Oxidative stress, which develops under hyperglycemia, alters the balance between pro- and anti-apoptotic proteins (*e.g.*, the Bcl-2 superfamily) in the cell and subsequently disrupts the mitochondrial trans-membrane potential and DNA structure[62].

THE USE OF BIOPREPARATIONS IN CURRENT APPROACHES OF DIABETES TREATMENT

Current approaches toward a potential cure for T1D have focused on three main targets: Ablation of the β-cell-specific autoimmune response; β-cell replacement therapy using islet transplantation; and potentiation of β-cell mass and function using pharmacologic agents capable of promoting β-cell proliferation, regeneration, and/or repair[63]. In the first method recombinant antigens that trigger an immune response of β-cell destruction are used. In this way, the tolerance of immune cells to auto-antigens is trying to be developed. However, this approach is quite new and not enough research has been conducted. The quickest and most promising approach that afforded patients complete independence from exogenous insulin is islet or β-cells' transplantation. However, it should be noted that this method is accompanied with intake of immunosuppressants to reduce the possibility of transplant rejection[63]. Therefore, at this stage of the fight against DM, most attention is

Table 1 Current oral anti-diabetic drug and their adverse effects

Types	Drug	Adverse effect
Sulfonylureas	Glibenclamide	Hypoglycemia, weight gain
Thiazolidinedione	Troglitazone	Liver damage
	Rosiglitazone	Cardiovascular disease
	Pioglitazone	Weight gain, pedal edema, bone loss, precipitation of congestive heart failure
α-glucosidase inhibitors	Migltol	Gastrointestinal effects (flatulence, diarrhea, stomachache)
	Acarbose	Gastrointestinal effects (flatulence, diarrhea, stomachache)
	Voglibose	Gastrointestinal effects (flatulence, stomachache)
Biguanide	Metformin	Gastrointestinal effects (diarrhea, vomiting, nausea)
	Phenformin	Lactic acidosis

paid to the study and research of drugs which are able to potentiate the effect of residual β-cells and/or induce their regeneration.

Several approaches were made to reduce the hyperglycemia, the hallmark of DM, with treatments such as sulfonylureas, which stimulate pancreatic islet cells to secrete insulin; meteoric, which acts to reduce hepatic glucose production; alpha-glucosidase inhibitors, which interfere with glucose adsorption and insulin itself, which suppresses glucose production and augments glucose utilization[64]. These therapies have limited efficacy, limited tolerability, and significant mechanism-based side effects (Table 1)[65]. Of particular danger is the use of some medicines that can promote weight gain, hypoglycemia, and insulin resistance. Another problem particular to some medicines (*e.g.*, sulphonylureas) is the development of resistance to the treatment. Therefore, the search for new preparations is extremely important. The growing public interest and awareness of natural medicines have led the pharmaceutical industry and academic researchers to pay more attention to medicinal plants and mushrooms. The apparent reversal of the trend from western to herbal medicine is partly due to the fact that synthetic drugs have always shown adverse reactions and other undesirable side effects. This has led to the belief that natural products are safer because they are more harmonious with biological systems[66,67]. In addition, the cost of administering modern antidiabetic drugs is beyond the reach of people in low-income groups and for those living in rural areas[68].

Medicinal mushrooms and their use

Mushrooms comprise an extremely abundant and diverse world. The number of mushroom species on Earth is currently estimated at 150000-160000; yet, perhaps only 10% are known to science[69]. Mushrooms are currently evaluated for their nutritional value and acceptability, as well as for their pharmacological properties[70]. They make up a vast and yet largely untapped source of new powerful pharmaceutical products. The majority of higher Basidiomycetes mushrooms contain many types of biologically active high-molecular weight and low-molecular weight compounds in fruit bodies, cultured mycelia, and cultured broth which has different properties[71,72].

Medicinal mushrooms have an established history of use in traditional oriental therapies. Contemporary research has validated and documented much of the ancient knowledge. Ancient oriental medicine has stressed the importance of several mushroom species, mostly *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst. (Ling zhi or Reishi) and *Lentinus edodes* (Berk.) Singer (Shiitake). Mushrooms have also played an important role as a cure for ailments affecting the rural populations of Russia and other Slavic European countries. The most important species in these areas were *Inonotus obliquus* (Pers.: Fr.) Pilat (Chaga), *Fomitopsis officinalis* (Vill.: Fr.) Bond. et Singer, and *Fomes fomentarius* Fr.: Fr. These species were used in the treatment of gastrointestinal disorders, various forms of cancers, bronchial asthma, night sweats, *etc*[73]. There is also a long history of traditional use of mushrooms as curatives in Mesoamerica (especially species of the genus Psilocybe), Africa (Yoruba populations in Nigeria and Benin), Algeria, and Egypt. A very special role of fly agaric [*Amanita muscaria* (L.:Fr.) Pers.] is found in Siberia and Tibetan shamanism, Buddhism, and Celtic myths[71,74,75].

Nowadays, medicinal mushrooms are used as dietary food, dietary supplemental products, pharmaceuticals, natural bio-control agents in plant protection with insecticidal, fungicidal, bactericidal, herbicidal, nematicidal, and antiphytoviral activities, and cosmeceuticals[72].

Medicinal mushrooms are comparable to "medicinal plants" and can be defined as macroscopic fungi, mostly higher Basidiomycetes and some Ascomycetes, which are used in the form of extracts or powder for prevention, alleviation, or healing of diseases, and/or in providing a balanced healthy diet[76].

Modern clinical practice in Taiwan, Japan, China, South Korea, and other Asian countries rely on mushroom-derived preparations. These preparations have many active compounds, which identify their importance as food and as medicines. This includes mainly high-molecular weight compounds such as polysaccharides (*e.g.*, β-D-glucans, glucuronoxylomannan), proteins, polysaccharide-protein complexes, lipopolysaccharides, glucoproteins, and lectins. Low-molecular weight metabolites include lactones, terpenoids, alkaloids, sterols, phenolic substances, and antibiotics with different active groups, metal chelating agents, *etc.* Also, medicinal mushrooms have enzymes - laccase, superoxide dismutase, glucose oxidase, peroxidase[72,77]. As dietary supplement products, mushrooms contain a small amount

of lipids and cholesterol, and low levels of carbohydrates. At the same time, they are rich in fiber, protein, minerals, and vitamins[78]. The wide range of bioactive compounds determines antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemic, antiviral, antibacterial, antiparasitic, hepatoprotective, and antidiabetic properties of medicinal mushrooms[79,80].

Antidiabetic properties of Agaricus brasiliensis and *Ganoderma lucidum*

Research has shown that some mushrooms may have the potential to lower elevated blood sugar levels. But the explanation for this effect is limited, except for some mushrooms. Therefore, it would be necessary to carry out more research on mushrooms with a focus to identify the active compounds in specific mushrooms for the treatment of DM and its complications.

Agaricus brasiliensis (*A. brasiliensis*, Royal Sun Agaricus) is native to Brazil and widely grown in Japan. This mushroom is used in the treatment of atherosclerosis, hepatitis, hyperlipidemia, dermatitis, and cancer, and its polysaccharides, α-glucan and β-glucan, have been shown to have immunomodulating and antimutagenic effects both *in vivo* and *in vitro*[75,81]. The possible mechanisms of natural polysaccharides to DM might base on six directions: (1) the elevation of plasma insulin, and the decline of pancreatic glucagon; (2) the increase of insulin sensitivity, and the improvement of insulin resistance; (3) the restraint of α-glycosidase enzymes in bowel, and the reduction of carbohydrates decomposition and absorption; (4) the increase of hepatic glycogen, and the inhibition of sugar dysplasia; (5) the increased glucose use of peripheral tissue; (6) the scavenging free radicals and lipid peroxidation[65]. Also, hypoglycemic and antidiabetic properties of *A. brasiliensis* have been reported. Di Naso *et al*[82] showed that *A. brasiliensis* extract exhibited a significant antioxidant activity in streptozotocin (STZ)-induced diabetic rats decreasing lipoperoxidation and iNOS expression in the lungs. These results suggest that *A. brasiliensis* treatment effectively reduced the oxidative stress and contributes to tissue recovery in diabetes. Another study demonstrated that *A. brasiliensis* extracts derived from submerged-culture broth significantly reduced blood glucose levels in an oral glucose-tolerant test in STZ-induced diabetic rats[83]. There is also clinical evidence that *A. brasiliensis* combined with antidiabetic drugs can improve insulin resistance in T2D patients[84]. It is shown that β-glucans and oligosaccharides of *A. brasiliensis* have antihyperglycemic, antihypertriglyceridemic, antihypercholesterolemic, and anti-arteriosclerotic activity in diabetic rats. One group has suggested that the *A. brasiliensis* antidiabetic effect in diabetic rats is due to its suppression of OS and proinflammatory cytokine production, which then results in improvement of the mass of pancreatic β-cells[85]. But, nevertheless, additional pharmacological studies are needed to elucidate the mechanism of *A. brasiliensis* action as well as to assess the use of these species for the treatment of human DM.

Ganoderma lucidum (*G. lucidum*, Ling zhi, Reishi), has a leading place in present-day medicinal mushroom development. *G. lucidum* has been utilized for centuries in East Asia to prevent or treat various diseases and was used in traditional Chinese medicine as a tonic in promoting good health, perpetual youth, vitality, and longevity. It is widely grown on a commercial scale and is commonly purchased for its medicinal and spiritual properties. Worldwide, more than 250 *Ganoderma* species have been described[75]. Recent studies on *G. lucidum* have shown many interesting biological activities including antitumor, antiinflammatory, antioxidant, and antidiabetic effects[75,77].

Antihyperglycemic effects of *G. lucidum* have been extensively studied and have shown potential therapeutic activities. It has been shown that oral administration of water extracts of *G. lucidum* significantly reduced the increase in blood glucose and insulin levels in rats following the oral glucose tolerant test[86]. Prevention of the progression of diabetic renal complications as well as a lowering of the increased serum glucose and triglyceride levels was reported in STZ-induced diabetic rats[87]. Another study demonstrated that polysaccharides isolated from *G. lucidum* significantly increased nonenzymatic and enzymatic antioxidants and serum insulin levels, and reduced lipid peroxidation and blood glucose levels in STZ-diabetic rats[88,89]. In alloxan-induced diabetic rats, the aqueous extract of *G. lucidum* normalized blood glucose levels[68].

It was shown that *G. lucidum* consumption can provide beneficial effects in treating T2D by lowering the serum glucose levels through the suppression of the hepatic enzyme gene expression involved in gluconeogenesis[90] in a clinical study showed that Ganopoly (polysaccharide fractions extracted from *G. lucidum* by a patented technique) efficaciously lowered blood glucose concentration in patients with confirmed T2D. It was shown that *G. lucidum* polysaccharides attenuated myocardial collagen cross-linking in diabetic rats, which was related to the decreased level of AGEs and augmented activities of antioxidant enzymes[91]. Also, it was shown that ganoderol B (bioactive sterol from *G. lucidum* fruit body) has a strong inhibitory activity on α-glucosidase and can be proposed as a treatment for T2D[92]. Orally administered proteoglycan extract, Fudan-Yueyang-*G. lucidum*, to STZ-induced diabetic rats showed a significant decrease in plasma glucose levels[93]. It appears that there are a number of biologically active compounds to be explored in the mycelium, and future research should focus in that direction.

RESULTS OF OUR RESEARCH

Correction of blood glucose and body weight of streptozotocin-induced diabetic rats

At present, more attention is paid to natural medicines which would have sugar lowering properties and are able to prevent angiopathy development, but, at the

same time, do not have adverse effects. There are significant evidences of the role of higher mushrooms in maintaining health and in disease prevention[94,95].

Our investigations showed significant increases (3.6 times) of blood glucose concentration in streptozotocin-induced diabetic rats compared with the control group. The per oral administration of submerged cultured mycelium powder (SCMP) during 14 d (at a dose of 1 g/kg of body weight) to control animals did not cause significant changes: Only slight fluctuations around values of control group. At the same time, administration of *A. brasiliensis* and *G. lucidum* SCMP to diabetic rats decreased blood glucose in 2.4 and 2.7 times, respectively, compared with values of the control diabetic group. It should be noted that obtained values were close to control ones[96].

During the research, we also measured the weights of animals because it characterized the general physiological state of the body. It is known that development of DM is accompanied by decreases in this index in sick animals[97]. Obtained results showed significant growth of body weight of control rats to the end of the experiment (by 18.3%) while body weight of diabetic animals significantly decreased (by 4.0%)[98]. As insulin plays the role of gluconeogenesis inhibitor, so deficit of insulin under DM causes intensification of this process. Thus, an increased blood glucose level is an inductor of different molecular and biochemical changes. Besides, removal of glucose from organism occurs with needed for this process amount of water and electrolytical ions K^+ and Na^+[99]. As a result, dehydration occurs in the organism. Further, dehydration of organism increasing due to the formation of ketone bodies (acetoacetate and β-hydroxybutyrate) and loss of bicarbonate blood buffer system capacity with the appropriate induction of ketoacidosis[100]. The administration of SCMP of medicinal mushrooms to control animals did not cause significant changes in body weight. In diabetic rats administered with *A. brasiliensis* and *G. lucidum* mycelium powder, we observed significant increases in this index by 4.0% and 8.5%, respectively[98].

Therefore, the use of medicinal mushrooms caused the lowering of blood glucose concentration and promoted body weight gain in diabetic rats. Such results indicate an overall protective effect of mushroom mycelium. The mechanism of such effects can be mediated through affecting blood glucose levels and the correction of ketone body content.

The content of glycosylated hemoglobin is another index that has been recently considered as a biomarker in the development and course of DM. It is believed to be a more precise glycemic indicator than blood glucose concentrations because HbA1c is more resistant to stress and independent of food intake[101]. During long-term hyperglycemia, the content of glycosylated hemoglobin increases due to intensification of non-enzymatic glycosylation processes[102]. Obtained results showed significant increases of HbA1c in streptozotocin-induced diabetic rats. Administration of SCMP of medicinal mushrooms to control groups led to a slight (not significant) growth of the index. In diabetic rats, administration of *A. brasiliensis* and *G. lucidum* mycelium powder caused significant reduction of HbA1c by 16.7% and 24.7%, respectively. This indicates a normalizing effect of the studied mushrooms on glucose metabolism[96].

Cytological indices of rats' peripheral blood

Inflammation and structural and functional disorders of the blood are additional risk factors involved in the pathogenesis of diabetes complications. Therefore, studying the impact of anti-diabetic drugs on morphological and functional parameters of blood cells is very important. It was shown that the development of diabetes occurs with a redistribution of segmented neutrophils and lymphocytes, and may indicate inflammatory processes in the animal under DM. Changes in the total number of white blood cells and their ratio, and violations in their functional properties are likely causes of susceptibility of patients with diabetes to infectious processes and immune status violations. For a preliminary assessment of the immune system of patients with diabetes type 1, the changes in white blood cell count were analyzed. There was no significant difference in the total number of leukocytes between the control group and the group with EDM, but the percentage of segmented neutrophils decreased by 27% and the percentage of lymphocytes increased by 8%, compared to the control[98].

Administration of mushroom mycelia in healthy animals did not lead to statistically significant changes in the ratio of different types of leukocytes. Under conditions of streptozotocin-induced DM, administration of mushroom SCMP resulted in normalization of the leukocyte formula, in particular, the increase in the number of segmented neutrophils. A decrease in the number of lymphocytes almost to the control values was also observed[98]. Thus, administration of powdered *A. brasiliensis* and *G. lucidum* to rats resulted in a normalization of the leukocyte formula.

Effect of mushroom powders on apoptosis of peripheral blood leukocytes in rats with streptozotocin-induced DM

Visual assessment of processes in leukocyte apoptosis by morphological features: The decrease of leukocyte membrane glycoconjugates sialylation and violation in the genetic apparatus of cells caused by oxidative-nitrative stress under DM may lead to cell death by apoptosis. Under apoptosis, the cell reduces its size; hardens its outer cytoplasmic membrane without the cell content entering the environment; aggregates chromatin and condenses the nucleus and cytoplasm; fragments them into vesicles surrounded by plasma membrane, *i.e.*, apoptotic bodies containing fragments of dying cells[103].

To quantify the content of apoptotic cells, the apoptotic index was calculated, *i.e.*, the ratio between cells with morphological apoptotic features and the general quantity of cells (Figure 1). Based on the collected data it was shown

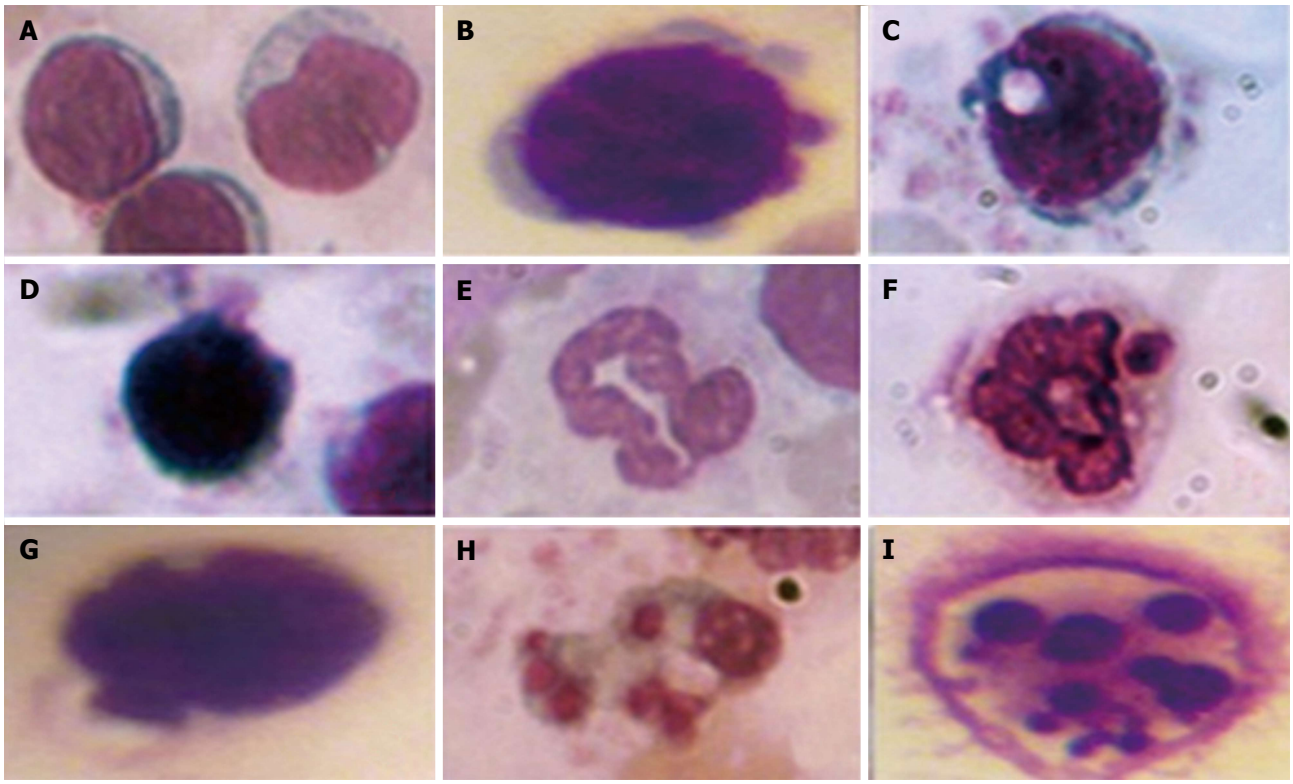

Figure 1 Morphological features of leukocytes apoptosis. Lymphocytes: Normal cell without apoptotic sings (A), zeiosis of the membrane (B), vacuolization of the cytoplasm (C), karyopyknozis of the nucleus (D). Neutrophils: Normal cell without apoptotic sings (E), vacuolization of the cytoplasm and the nucleus (F, G), vacuolization of the cytoplasm (H) and karioreksis of the nucleus (I). In smears stained by the Romanovsky-Himza method, the number of white blood cells with features of apoptosis was assessed. The ratio between cells with morphological apoptotic features and the general quantity of cells were expressed in percentages.

that the development of diabetes was accompanied by an 8-fold increase of the apoptotic index. Whereas administration of *A. brasiliensis* and *G. lucidum* to diabetic animals led to a reduction in the apoptotic index by 3.5-fold and 3.8-fold, respectively (compared with non-treated diabetic animals)[98].

Immunocytochemical detection of the content of apoptotic protein-regulators (p53 and Bcl-2): The p-53 protein has a special place in the regulation of apoptosis. Under stress, the p-53 protein can induce apoptosis in response to numerous adverse effects leading to a variety of genetic disorders[62]. This process occurs through Bcl-2 protein inactivation by binding to Bax. As a result, a heterodimeric complex is formed, which stops the anti-apoptotic activity of the Bcl-2 protein. We showed that under streptozotocin-induced DM the number of $p53^{++}$ cells (high-positive response) increased by 47%, and the number of $p53^{+}$ cells (positive response) increased by 32% compared with the control, whereas the number of $p53^{-}$ cells (negative reaction) decreased by 25%[98] (Figure 2). Administration of selected mushroom SCMP in control animals revealed no significant changes. The administration of the mushrooms led to a decrease in the level of $p53^{++}$ and $p53^{+}$ cells in diabetic animals compared to untreated diabetic animals. Under *A. brasiliensis* administration, there was a decrease of 22% in the level of $p53^{++}$ and 44.7% in the level of $p53^{+}$. Also, our results have shown a significant increase in the number of cells with a p53 negative reaction by 20%. Administration of *G. lucidum* mycelium powder led to a decrease in the number of cells with positive response by 40.5%, cells with high-positive response by 47%, and increased the number of cells with a p53 negative reaction by 27.6% compared to untreated animals[98].

There are regulators that block or magnify destructive effects of caspases. Proteins from the Bcl-2 family belong to this group. Different members of the family can form dimers with each other, one of them enhancing or inhibiting the function of the other. In this case, the ratio between inhibitors and activators may show the disposition of cells to apoptosis[104]. Particular interest here is displayed in Bcl-2 protein. It is known that Bcl-2 inhibits p53-dependent and -independent ways of apoptosis[56].

We have shown that development of DM was accompanied by a increase in the content of $Bcl\text{-}2^{+}$ and $Bcl\text{-}2^{++}$ cells and a decrease in the content of $Bcl\text{-}2^{-}$ cells (Figure 3). Administration of studied mushroom SCMPs to control animals did not cause any significant changes. Administration of *A. brasiliensis* and *G. lucidum* to the diabetic animals caused the normalization in the percentage of cells with different levels of Bcl-2 protein expression compared to the control values[98].

Thus, these results suggest an increase of apoptotic processes in the peripheral blood leukocytes of rats with streptozotocin-induced DM. Effects of medicinal mushrooms under conditions of diabetes are aimed at

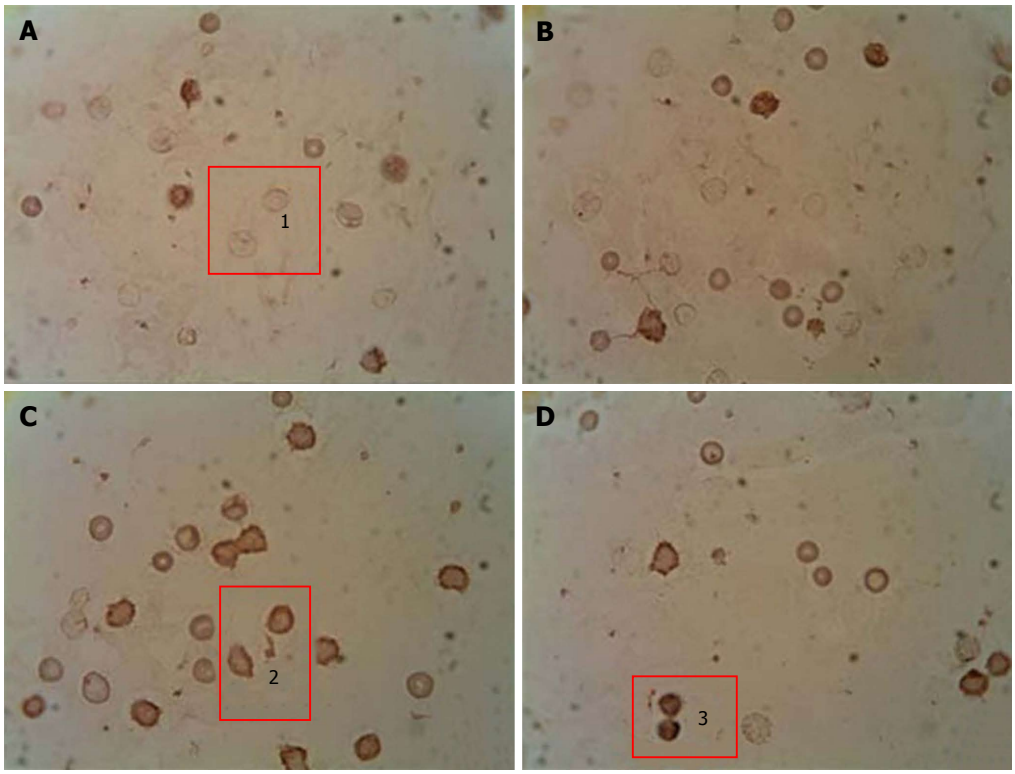

Figure 2 Immunocytochemical analysis of peripheral blood leukocytes in rats depending on the content of p53 pro-apoptotic protein in healthy animals, animals with streptozotocin-induced diabetes and treated with submerged cultured mycelium powder of mushrooms. A: Control; B: Control animals treated with *Agaricus brasiliensis* (*A. brasiliensis*); C: Animals with STZ-induced diabetes mellitus; D: Diabetic animals treated with *A. brasiliensis*. 1: $p53^{-}$; 2: $p53^{+}$; 3: $p53^{++}$. An indirect immunoperoxidase method was used for detection and visualization of intracellular protein p53. The content analysis of p53 in leukocytes of rat peripheral blood was performed by light microscopy using a × 40 microscope objective. Depending on intensity of staining, the cells were divided into 3 groups: Negative reaction ($p53^{-}$), positive reaction ($p53^{+}$), and high-positive ($p53^{++}$) reaction.

normalization of the ratio of WBCs that contain proteins-regulators of apoptosis (p53 and Bcl-2) as well as reducing apoptotic index. This indicates a pronounced anti-apoptotic effect of the studied species of mushrooms.

Changes in blood erythroid link: Since hyperglycemia might cause much damage in membrane components of RBC, it can affect their number in the bloodstream. This in turn might lead to impairment of their functions. Therefore, we paid attention to the quantity of characteristics of the blood. Our research showed that the number of red cells significantly decreased in streptozotocin-induced diabetic rats[96]. Such results indicate the development of anemia that accompanies DM and is one of the causes of tissue hypoxia and secondary complications. In control animals administered with medicinal mushroom mycelium powder, we observed a tendency to decrease the RBC number, but obtained changes were not significant. In diabetic SCMP-treated rats the number of red cells significantly increased compared with non-treated animals, and the values almost reached the level of controls[96].

The impairment of RBC membrane structures in peripheral blood caused by chronic hyperglycemia and insulin deficiency leads to changes in the functional state of the cells. One of the methods used for its evaluation is RBC resistance to acid hemolysis. The method of acidic erythrograms helps to estimate the value of the hydrophobic barrier and permeability of membrane protein components, and provides an opportunity to group morphologically similar RBCs into age populations. Young red cells possess the highest resistance to acid hemolysis and take erythrograms correctly. The aging of RBCs is accompanied by a gradual decrease of their resistance to acidic hemolysis, which is reflected in an erythrogram shift to the left. Its level reveals the functional-metabolic state of the cell membrane and undergoes changes not only due to cell aging but also as a result of physicochemical modifications and destabilization of membrane components under various pathologies[105].

Results of the investigation of RBCs' resistance to acid hemolysis are described in our previous paper[96]. Development of DM in rats is accompanied by the shift of erythrogram to the left, reduction of hemolysis time, the accelerated peak of hemolysis and higher percentage of hemolyzed RBCs at the peak's point. A decrease in red cell resistance to acid hemolysis indicates not only the aging of the cells but also reveals the possibility of their membrane destruction during formation and maturation.

Administration of SCMP of medicinal mushrooms to the control group did not cause changes in the parameters of hemolysis. However, in diabetic rats this administration led to the return of the above mentioned parameters to control values. Obtained results indicate the redistribution of RBC populations towards the growth of

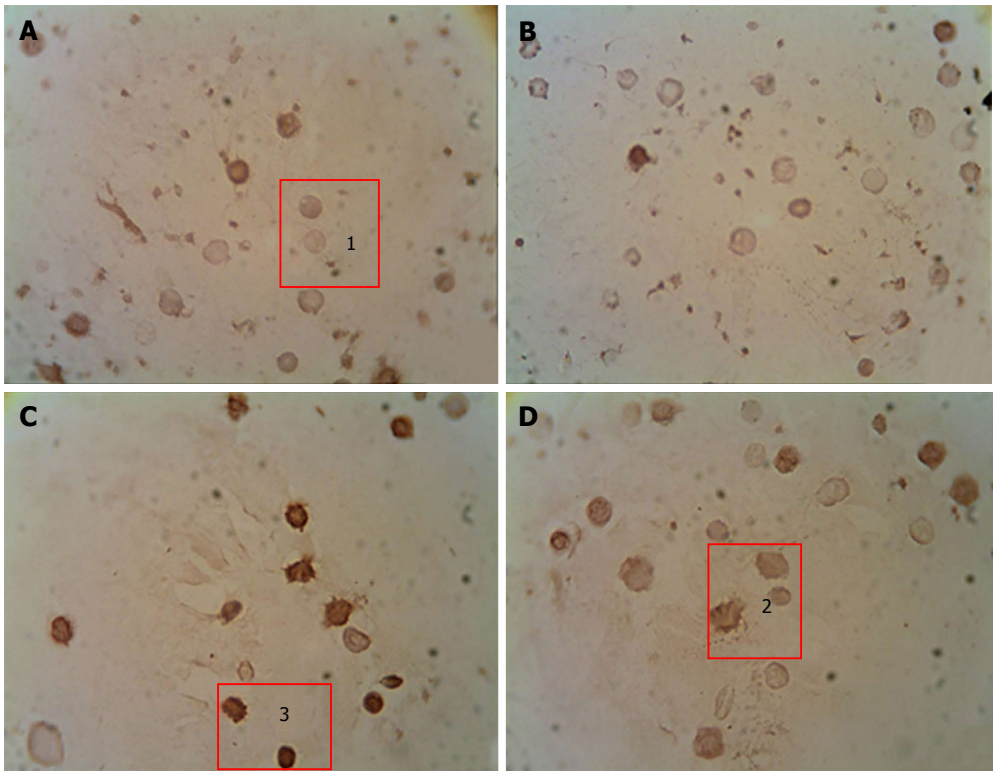

Figure 3 Immunocytochemical analysis of peripheral blood leukocytes in rats depending on the content of anti-apoptotic Bcl-2 protein in healthy animals, animals with streptozotocin-induced diabetes and treated with the submerged cultured mycelium powder of mushrooms. A: Control; B: Control animals treated with *G. lucidum*; C: Animals with STZ-induced DM; D: Diabetic animals treated with *G. lucidum*. 1: Bcl-2^{-}; 2: Bcl-2^{+}; 3: Bcl-2^{++}. An indirect immunoperoxidase method was used for detection and visualization of intracellular proteins Bcl-2. The content analysis of Bcl-2 in leukocytes of rat peripheral blood was performed by light microscopy using a × 40 microscope objective. Depending on intensity of staining, the cells were divided into 3 groups: Negative reaction (Bcl-2^{-}), positive reaction (Bcl-2^{+}), and high-positive (Bcl-2^{++}) reaction. *G. lucidum*: *Ganoderma lucidum*; STZ: Streptozotocin; DM: Diabetes mellitus.

full functional cell numbers, which confirms a corrective membrane protective effect produced by the selected mushrooms[96].

Reduction of the RBCs' number and an increased content of HbA1c lead to a violation in tissue oxygenation and the development of hypoxia. The body launches compensatory reactions aimed at restoring homeostasis[105]. One such reaction under conditions of DM is an intensification of erythropoiesis that can be assessed by the determination of number and daily production of reticulocytes - precursors of mature RBCs. Our study showed that the development of diabetes intensified erythropoiesis in rats evidenced by the increased number of reticulocytes in the bloodstream and their daily production[96]. *A. brasiliensis* treatment of control animals led to significant increases of the precursors' number and their production. At the same time, there were no changes of these indices detected in the *G. lucidum* treated group[90]. In diabetic rats, administration of medicinal mushrooms' SCMP did not affect erythropoiesis effectiveness, *i.e.*, elevated levels remained[96].

BIOLOGICAL ACTIVE COMPOUNDS RESPONSIBLE FOR GLYCEMIC CONTROL

The reduced levels of blood glucose and content of glycosylated hemoglobin after the course of medicinal mushroom mycelium powders administration were shown on the model of rats with streptozotocin-induced DM. It is believed that the major bioactive compounds of *A. brasiliensis* are polysaccharides and protein-polysaccharide complexes containing beta-glucans[81,106]. The effect of beta-glucans to reduce blood glucose could be mediated possibly by delaying stomach emptying, so that dietary glucose is absorbed more gradually[105]. Tapola *et al*[107] showed that after ingestion of oats (with high content of beta-glucans), the blood glucose levels were lower at 15, 30 and 45 min but higher at 90 min after 12.5 g glucose loading. Thus, the peak level is much more smoothed and the shape of the plasma glucose response curve is much flatter[107]. Another possible mechanism for beta-glucans is mediated by a signal pathway through activation of PI3K/Akt in which activity decreases under DM[108]. Beta-glucans interact with a specific receptor and, through messenger molecules [*e.g.*, SYk kinase[109]], activate the PI3-kinase pathway. Bioactive compounds may also include oligopeptides. Recently described oligopeptide of *A. brasiliensis* is rich in proline, lysine, and phenylalanine and has antioxidant activity[110]. Also, *A. brasiliensis* contains low-molecular weight compounds such as tocopherol, ergosterol, phenols, *etc.*[111,112] and metal ions[85,113]. These compounds are included in different regulated mechanisms responsible for inhibition of free radicals formation and the

development of oxidative stress. Microelements promote the function of the antioxidant defense system. They include beta-carotine, vitamin C, vitamin E (the vitamin E family includes both tocopherols and tocotrienols, but alpha-tocopherol is predominant and the most active form). Water-soluble molecules, such as vitamin C, are powerful radical scavengers in the aqueous phase of cytoplasm, while lipid-soluble molecules (such as vitamin E and beta-carotene) are antioxidants in the lipid phase. Metal ions (*e.g.*, selenium, copper, zinc, manganese, *etc.*), besides scavenger properties, are in active sites of enzymes of the antioxidant system[114]. In addition, three phenols - gallic acid, lilac acid, and pirogallol, which have pronounced antioxidant activities, were identified in *A. brasiliensis*[111]. Such a protective effect, in turn, improves functioning of residual pancreas beta cells and improves insulin secretion[85].

The major active components of *G. lucidum*, which have antidiabetic properties, are polysaccharides and triterpenoids[90]. The therapeutic effect of polysaccharides consists of: The facilitation of glucose uptake by muscle cells through activation of PI3K and AMPK-dependent metabolic pathways[115]; regulation of the expression of key enzymes in the conversion of glucose (*e.g.*, liver glucokinase, phosphofructokinase, glucose 6-phosphate dehydrogenase, *etc.*)[116,117]; and an increase of insulin content in blood plasma[68]. Triterpenoids show aldose reductase and α-glucosidase inhibitory properties that correlate with glucose metabolism and diabetic complications[118]. Another mechanism of the protective effect of this mushroom is the scavengering of free radicals and thus "neutralize" the negative impact of oxidative stress (on β-cells of the pancreas, in particular), which is one of the etiological causes of diabetes development[88].

In addition, medicinal mushrooms are rich in nucleotides and nucleozides, *e.g.*, adenosine, which (in the same way as A1 activators of purinergic receptors) shows cytoprotective action in cardiovascular and central nervous systems by activating adenosine receptors of the cell surface. Activation of these receptors, in turn, activates antioxidant enzymes. This occurs *via* the protein kinase C, which phosphorylates those enzymes or intermediates that contribute to their activation[119].

CONCLUSION

Despite the great efforts made in the field of DM treatment, this disease is still one of the major problem of humanity. Physical and biochemical changes of blood cells, that occurred due to metabolic disorders, lead to the development of different kinds of angiopathies and the worsening of patients' health. Medicinal mushrooms *A. brasiliensis* and *G. lucidum*, due to their composition and biological properties, are potential sources of new biopreparations for the prevention and treatment of DM. SCMP of medicinal mushrooms has a pronounced hypoglycemic effect manifesting in the normalization of blood glucose levels and content of glycosylated hemoglobin. Also, mycelium powders promote weight gain of rats with STZ-induced diabetes confirming the general restorative role for the body. The selected mushrooms showed the normalizing effect on the indices of leukocyte formula under DM type 1. However, they do not show a significant impact on the number of leukocytes in healthy animals, which indicate no adverse physiological effects of their action. The action of the medicinal mushrooms under diabetes is aimed at the normalization of a ratio between protein-regulators of apoptosis (p53 and Bcl-2) in white blood cells, as well as the reduction of the apoptotic index. These changes indicate an inhibitory effect of the selected mushrooms on programmed cell death, which is significantly increased under DM. Administration of medicinal mushrooms *A. brasiliensis* and *G. lucidum* leads to restoration of RBCs' number and their resistance to acid hemolysis indicating the anti-anemic and membrane protective impacts.

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ORIGINAL ARTICLE

Clinical Trials Study

Association between dairy intake, lipids and vascular structure and function in diabetes

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Abstract

AIM

To determine lipid species that change in response to a change in dairy consumption. In addition, to investigate whether dairy associated lipid species are correlated with changes in measures of vascular structure and function.

METHODS

A 12-mo randomised controlled trial was conducted to determine the effect of increased consumption of fruit, vegetables and dairy, compared to usual diet, on measures of vascular structure and function in adults with type 1 and type 2 diabetes (n = 108). This

paper comprises *post-hoc* analyses investigating the relationship between dairy intake, serum lipid species and vascular health. Central and peripheral blood pressure, carotid femoral pulse wave velocity, augmentation index, serum lipid species and dietary intake were measured at baseline and 3-mo. Common carotid artery intima media thickness was measured at baseline and 12-mo.

RESULTS

Serum lipid species [lysophosphatidylcholine (LPC) 14:0, LPC 15:0, LPC 16:1, phosphatidylcholine (PC) 29:0 PC 30:0, PC 31:0 and cholesterol ester (CE) 14:0] were associated with the change in full fat dairy consumption (rho 0.19-0.25; $P < 0.05$). The 3-mo change in some lipids was positively associated with the 3-mo change in central systolic [LPC 14:0 (rho 0.30; $P = 0.007$), PC 30:0 (rho 0.28; $P = 0.010$)] and diastolic blood pressure [LPC 14:0 (rho 0.32; $P = 0.004$), LPC 15:0 (rho 0.23; $P = 0.04$), LPC 16:1 (rho 0.23; $P = 0.035$), PC 29:0 (rho 0.28; $P = 0.01$), PC 30:0 (rho 0.36; $P = 0.001$), PC 31:0 (rho 0.30; $P = 0.007$)] and 12-mo change in common carotid artery intimal medial thickness [CE 14:0 (rho 0.22; $P = 0.02$)]. Pulse wave velocity and augmentation index were unrelated to dairy and lipid species.

CONCLUSION

An increase in dairy associated lipids appears to be associated with an increase in blood pressure and common carotid intimal medial thickness.

Key words: Lipids; Phospholipids; Atherosclerosis; Dairy; Lysophosphatidylcholine; Lipidomics; Carotid intima media thickness; Pulse wave velocity; Diabetes

Core tip: We have examined the relationship between changes in dairy intake, lipid species and vascular function. Although it was expected that an increase in dairy intake would lower blood pressure and be associated with improvements in vascular structure we found that increases in lipid species associated with dairy (LPC 14:0, LPC 15:0, LPC 16:1, CE 14:0) were associated with adverse changes in these parameters. Dairy does not appear to be beneficial in people with diabetes.

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INTRODUCTION

By 2030 it is projected that 7.7% of the world's population will have diabetes, which is an increase of 54% since 2010[1]. Individuals with type 1 and type 2 diabetes are two-to-three times more likely to develop cardiovascular disease (CVD) compared with the general population[2-4]. In Australia, in 2010 approximately 30% of all deaths in people with type 1 and type 2 diabetes were due to CVD[5]. Poor diet is the leading contributor to the global burden of disease[6] and better dietary quality is associated with lower rates of CVD[7,8]. The most recent evidence from prospective cohort studies suggests that dairy consumption is protective against CVD[9]. Although uncertainty remains about the vascular effects of dairy fatty acids[10].

Arterial stiffness measured by augmentation index and carotid femoral pulse wave velocity (cfPWV) are independent predictors of CVD[11,12]. Similarly, carotid intima media thickness (IMT) is an early measure of atherosclerosis that predicts CVD[13,14]. Epidemiological studies have indicated that higher consumption of dairy products is associated with lower carotid IMT[15,16] and less arterial stiffening[15,17-19].

Self-reported dietary intake is limited by inaccurate reporting, which is well-documented in the general population and people with diabetes[20]. Biomarkers of dietary intake, including dairy consumption, remove the reliance on self-reported dietary data. Previously it has been shown that fatty acids of ruminant origin are correlated with self-reported dietary intake of dairy products[19,21].

The aim is to determine serum lipid species that change in response to a change in dairy consumption. Based on our previous analysis[19] we hypothesise that the following lipid species will reflect a change in dairy consumption: Cholesterol ester (CE) 14:0, CE 15:0, lysophosphatidylcholine (LPC) 14:0, LPC 15:0, LPC 17:1, phosphatidylcholine (PC) 29:0, PC 30:0, PC 31:0, PC 31:1, PC 33:0, PC 33:1, PC 33:3, PC 35:0, sphingomyelin (SM) 31:1, SM 32:0, triacylglycerol (TG) 16:0/16:0/16:0. The secondary aims are to determine the correlation between the change in dairy associated lipid species and 3-mo change in peripheral and central blood pressure, augmentation index and cfPWV. The 12-mo change in common carotid artery intima media thickness (CCA-IMT) will also be correlated with the change in lipid species.

MATERIALS AND METHODS

Study design

As previously reported, a 12-mo randomised controlled trial was conducted to determine the effect of improving dietary quality on CCA-IMT, compared to a control group continuing on their habitual diet, in a cohort of people with type 1 and type 2 diabetes[22]. Briefly, participants were randomised to increase consumption of fruit (+1 serve/day), vegetables (+2 serves/day) and dairy (+1 serve/day), regardless of usual intake or to continue on their usual diet. One serve of dairy was either 250 mL of milk or 200 g of yoghurt and no advice was given regarding the fat content. Dietary counselling was provided by a dietitian. Ethics approval was obtained from the University of South Australian Human Research Ethics Committee

and the participants provided written informed consent. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12613000251729) on 04/09/2014.

A post hoc analysis was conducted that comprised a subsample of the cohort (n = 108) that had serum lipidomic analysis performed at baseline and 3-mo. A food frequency questionnaire (FFQ) was used to measure dietary intake at baseline and 3-mo and a fasting serum sample was collected for measurement of lipid species. Participants had peripheral and central blood pressure, augmentation index and cfPWV measured at baseline and 3-mo. In addition, CCA-IMT was measured at baseline and 12-mo. The intervention to increase dairy by one serve per day was only partially successful with 34 people in the intervention group increasing and 17 decreasing their dairy consumption, while in the control group 23 increased and 34 reduced their dairy intake. Thus overall 57 people increased and 51 decreased dairy intake so the analyses were performed without regard for allocation to intervention or control.

Subjects

Subjects above 18 years of age with diagnosed type 1 or type 2 diabetes for any duration managed with diet, oral hypoglycaemic agents (OHA) and/or insulin were recruited from August 2012 until December 2013 from a database of volunteers, public advertisements and a recruitment company (Intuito Market Research, Adelaide, South Australia). Exclusion criteria were: Unstable CVD requiring active intervention, heart failure, significant renal impairment (eGFR < 30 mL/min), liver disease, cancer or allergic/intolerant/dislike of fruit, vegetables or dairy.

Serum lipid analysis

Lipid analysis was performed on fasting serum by liquid chromatography, electrospray ionization-tandem mass spectrometry as previously published[23]. In total, 342 individual lipid species were measured from 23 classes.

Food frequency questionnaire

Habitual dietary intake was measured using the electronic version of the Dietary Questionnaire for Epidemiological Studies Version 2 FFQ. The FFQ includes questions on the types of milk (fat content), cheese (hard, firm, soft, ricotta, cottage cheese, cream cheese or low fat cheese) and spreads (butter) used. Questions about the quantity of milk consumed per day (none, < 250 mL, 250-500 mL, 500-750 mL, > 750 mL) and the frequency of cheese, yoghurt and ice-cream consumption are also included. This FFQ has been found to have relatively good agreement with a 3-d weighed food record in the general population[24] and in people with type 1 and type 2 diabetes[25].

Measurements

Anthropometric measurements: Height was measured using a stadiometer (SECA, Hamburg, Germany) to the nearest 0.1 cm while barefoot/flat footwear. Weight was measured to the nearest 0.05 kg using calibrated electronic scales (SECA, Hamburg, Germany) while the participants were barefoot/light footwear and wore light clothing.

Peripheral blood pressure: Clinic blood pressure was measured using an automated sphygmomanometer (SureSigns VS3; Philips, North Ryde, Sydney, Australia) once the participant had been seated for 5 min. A normal sleeve (16 cm × 52 cm) was used for an arm circumference of 24-32 cm and a large sleeve (16 cm × 70 cm) for an arm circumference of 32-42 cm. A minimum of four consecutive readings were taken at 1 min intervals. The first reading was discarded and the following three consistent measurements, *i.e.*, systolic blood pressure within a range of 1.3 kPa, were used.

Common carotid artery intima media thickness: The measurements of the carotid artery were taken using B mode ultra-sound by one operator, with an intra-observer coefficient of variation (CV) of 4.4% (n = 34). The participants were supine with their head positioned at 45 degrees away from the side of the neck being measured. A high resolution ultrasound machine with a 12 MHz transducer was used (Samsung Medison MySono U6, South Korea). A 1 cm region of the IMT on the far wall of the distal common carotid artery on both sides was measured using automatic edge detection software (Samsung Medison MySono U6 Auto IMT, South Korea) as recommended in the Mannheim Carotid Intima-Media Thickness Consensus Paper (2004-2006-2011)[26]. Areas of plaque, defined as a 50% greater IMT than the surrounding IMT or IMT > 1.5 mm, were not imaged. Three seconds clips were captured and the mean of 10 measurements taken from each of these clips was averaged for a mean and mean maximum CCA-IMT value at baseline and 12 mo.

Central blood pressure and augmentation index: A SphygmoCor® XCEL (AtCor Medical, West Ryde, Sydney, Australia) was used to measure central blood pressure and augmentation index. A cuff was placed over the brachial artery on the right arm and measurements were completed after the participants had been quietly resting for 5 min. Three consecutive measurements were taken and the average calculated. All of the measurements were taken by one operator with a CV of 4.2% (n = 28).

Pulse wave velocity: A SphygmoCor® XCEL (AtCor Medical, West Ryde, Sydney, Australia) was used to measure cfPWV. The tonometer was placed on the right carotid artery and the cuff on the right femoral artery. A 10-s recording of the carotid-femoral waveform was taken. Three measurements were performed at each time-point and an average taken. The measurements

were taken by two operators; the intra-observer CVs were 4.2% (n = 28) and 7.3% (n = 11), respectively and the inter-observer CV was 5.0% (n = 18).

Statistical analysis

Data are presented as mean ± SD. The change in dairy intake between baseline and 3-mo was not different between the groups so the cohort was analysed as a whole. Paired samples *t*-tests were used to determine the change in dairy intake, anthropometric measures, blood pressure, biochemistry and vascular measurements over time.

Change in lipid species containing fatty acids 15:0, 16:1 and 17:0 known to be of ruminant origin[27-29] (CE 15:0, CE 16:1, CE 17:0, diacylglycerol (DG) 16:1/18:1, LPC 15:0, LPC 16:1, LPC 17:0, TG 15:0/18:1/16:0, TG 15:0/18:1/18:1, TG 16:1/16:1/16:1, TG 16:1/16:1/18:0, TG 16:1/16:1/18:1, TG 16:1/18:1/18:1, TG 16:1/18:1/18:2, TG 17:0/16:0/16:1, TG 17:0/16:0/18:0, TG 17:0/18:1/14:0, TG 17:0/18:1/16:0, TG 17:0/18:1/16:1, TG 17:0/18:1/18:1, TG 17:0/18:2/16:0) and those we have previously shown to be correlated with dairy consumption[19] (CE 14:0, CE 15:0, LPC 14:0, LPC 15:0, LPC 17:1, PC 29:0, PC 30:0, PC 31:0, PC 31:1, PC 33:0, PC 33:1, PC 33:3, PC 35:0, SM 31:1, SM 32:0, TG 16:0/16:0/16:0) were correlated with the 3-mo change in full fat dairy consumption. Spearman's correlation was used to correlate the change in dairy consumption with the change in lipid species because the data did not have a normal distribution. Lipid species that were correlated with the change in full fat dairy consumption were correlated with the change in vascular measurements. Both absolute and percent change (change/baseline × 100) are presented. No adjustment for multiple testing were made with this analysis. Secondary analyses involving all of the lipid species (> 300) were conducted. Spearman's correlation was used to determine the association between the change in lipid species and the change in vascular measures. In addition, baseline concentrations of the lipid species were correlated with the change in vascular measures. These secondary analyses were corrected for multiple comparisons using the Benjamini Hochberg approach[30]. Analyses were performed using SPSS (version 19, 2010, SPSS Inc, Chicago, IL, United States). Statistical significance was set at $P < 0.05$. The statistical methods of this study were reviewed by Ms Kylie Lange from the University of Adelaide, Australia.

RESULTS

A total of 108 participants were involved in these analyses (Figure 1). Baseline characteristics of the participants included in this subsample were not different from non-participants with regards to age, weight, BMI, peripheral blood pressure, central systolic blood pressure, mean or mean maximum CCA-IMT. Central diastolic blood pressure, augmentation index, cfPWV and HbA1c were higher ($P < 0.05$) in the non-participants compared with the participants. Baseline characteristics of the participants are presented in Table 1.

Table 2 shows consumption of dairy products measured using the FFQ. There was no significant change in total or full fat dairy consumption over time. Yoghurt consumption increased while cheese and butter consumption decreased. There was no effect of the treatment on dairy consumption or lipid levels, so we were able to examine the association between the change in dairy intake and the change in lipid species in the entire cohort to identify dairy associated lipid species. And we further analysed the correlation between the change in dairy associated lipid species and vascular measurements.

Table 3 shows the results of correlating the change in lipid species containing 15:0, 16:1 or 17:0 or those identified as correlating with dairy intake in the previous cross-sectional study with the change in full fat dairy consumption (milk, yoghurt, cheese, butter and ice-cream). No adjustments were made.

Table 4 shows the anthropometric measurements, blood pressure, biochemistry and vascular measurements at baseline and follow-up in the whole group. Measurements of central blood pressure and augmentation index were performed on 82 participants due to equipment availability. cfPWV was performed on 70 participants due to technical difficulties because of obesity. The lipid species that were shown to be correlated with total full fat dairy consumption (LPC 14:0, LPC 15:0, LPC 16:1, PC 29:0, PC 30:0, PC 31:0, CE 14:0) were then correlated with the vascular measurements that changed over time.

Table 5 presents the results of these analyses including both absolute and percent change in each parameter. Percent and absolute change in LPC 14:0, PC 30:0 and PC 31:0 were correlated with percent and absolute change in central systolic and diastolic blood pressure and central mean arterial pressure. Change in central diastolic blood pressure was correlated with the change in LPC 15:0, LPC 16:1, PC 29:0 and PC 31:0. Change in central mean arterial pressure was also correlated with the change in PC 31:0. The absolute and percent change in CCA-IMT was correlated with the absolute and percent change in CE 14:0 only. There were no lipid species that were associated with the change in augmentation index or cfPWV. Change in full fat dairy consumption itself was not associated with the change in any of the vascular measurements. When the change in all of the lipid species (n > 300) was examined after correction for multiple comparisons, there were no significant correlations between any of the lipid species and the change in central systolic and diastolic blood pressure, central mean arterial pressure, augmentation index or CCA-IMT.

Baseline concentration of PC 34:4 (rho = -0.32; $P < 0.0001$), PC 32:2 (rho = -0.33; $P < 0.0001$), G_{M3} ganglioside (GM3) 16:0 (rho = -0.34; $P < 0.0001$), GM3 18:0 (rho = -0.38; $P = 0.0001$) and total GM3 (rho

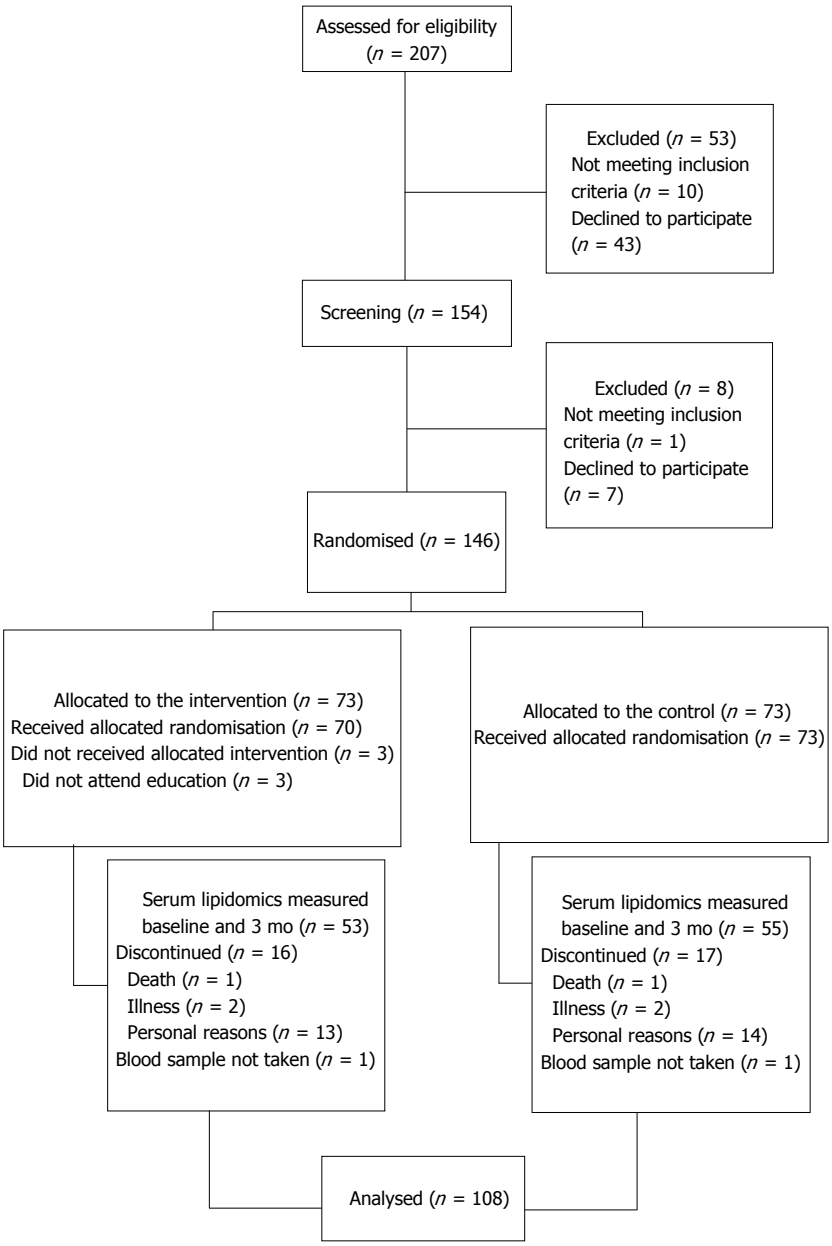

Figure 1 CONSORT diagram.

= -0.36; *P* = 0.0003) were inversely associated with the change in CCA-IMT, after adjustment for multiple comparisons. The change in central systolic and diastolic blood pressure, central mean arterial pressure, augmentation index or cfPWV were not associated with the concentration of any lipid species at baseline after correction for multiple comparisons.

DISCUSSION

These analyses show that a change in the concentration of a number of serum lipid species (LPC 14:0, LPC 15:0, LPC 16:1, PC 29:0 PC 30:0, PC 31:0, CE 14:0), previously shown to be correlated with dairy consumption, were associated with a change in full fat dairy consumption in the present study. One or more of these lipid species were positively associated with the 3-mo change in central blood pressure and 12-mo change in CCA-IMT in this cohort with type 1 and type 2 diabetes.

Fatty acids C 15:0 and C 17:0 are not produced endogenously and are constituents of dairy fat. The fatty acid C 14:0 is produced endogenously but is also a component of dairy fat. Therefore these fatty acids in plasma or for C 14:0 in adipose tissue are considered biomarkers of dairy intake[28]. In the present study change in LPC 15:0, LPC 14:0 and CE 14:0 were weakly positively correlated with the change in consumption of full fat dairy. LPC concentration in milk fat is < 1% of the total PC concentration and therefore dairy would be an unlikely direct source of LPC species[21]. LPC lipid species are predominately produced endogenously through a number of different processes including from

Table 1 Baseline characteristics of the participants *n* (%)

Characteristic	Cohort (*n* = 108)
Age (yr)	58 ± 14
Weight (kg)	96.2 ± 20.6
BMI (kg/m^2)	32.6 ± 6.4
Sex	
Female	40 (37)
Male	68 (63)
Diabetes type	
Type 1	14 (13)
Type 2	94 (87)
Time since diabetes diagnosis (yr)	
Type 1	21 ± 13
Type 2	8 ± 7
Smoking status	
Never smoked	56 (52)
Past smoker	47 (43)
Current smoker	5 (5)
Smoking pack years (years)[a]	10.5 ± 16.3
Prescribed anti-hypertensive medication	66 (61)
Prescribed lipid lowering medication	61 (57)
Diabetes treatment	
None	22 (20)
OHA	49 (46)
Insulin	16 (15)
OHA + insulin	21 (19)
Presence of Microalbuminuria[1]	14 (13)
HbA1c	7.1 ± 1.2

All values are mean ± SD or *n* (%) where specified; [a]Smoking pack years = number packs (25 cigarettes)/day × number years smoked; [1]Albumin: creatinine > 2.5 for men and > 3.5 for women. BMI: Body mass index; OHA: Oral hypoglycaemic agents.

Table 2 Dietary intake measured using a food frequency questionnaire

Intake (g/d)	Cohort (*n* = 108)		*P* value for change
	Baseline	3 mo	
Total dairy[1]	432 ± 218	434 ± 198	0.90
Total full fat dairy[1]	113 ± 201	94 ± 162	0.16
Milk	349 ± 201	345 ± 188	0.78
Yoghurt	55 ± 65	68 ± 76	0.031
Cheese	14 ± 12	11 ± 10	0.005
Butter	2 ± 6	1 ± 4	0.051
Ice-cream	11 ± 18	9 ± 10	0.063

[1]Includes milk, yoghurt, cheese, butter, ice-cream. All values are mean ± SD; Data analysed using paired samples *t*-test.

Table 3 Spearman's correlation of the change in total full fat dairy (milk, yoghurt, cheese, butter, ice-cream) with change in lipid species

Change in lipid species	rho value	*P* value
LPC 14:0	0.25	0.01
LPC 15:0	0.21	0.03
LPC 16:1	0.22	0.02
PC 29:0	0.22	0.02
PC 30:0	0.23	0.02
PC 31:0	0.19	0.049
CE 14:0	0.24	0.01

LPC: Lysophosphatidylcholine; PC: Phosphatidylcholine; CE: Cholesterol ester.

PC by phospholipase A_2 enzyme and the oxidation of LDL particles[31,32].

In a randomised cross-over study, consumption of a full fat dairy diet containing non-fermented products (butter, cream or ice cream) was associated with higher plasma concentrations of sphingomyelin compared with low-fat dairy intake[33]. Plasma concentrations of odd chain phosphatidylcholine (15:0 and 17:0) were increased with consumption of both full fat fermented (including yoghurt and cheese) and non-fermented dairy products. In this study vascular measurements were not performed. Previously we showed a cross-sectional association between serum lipid species and consumption of full fat dairy in people with type 1 and type 2 diabetes but serum lipid species were not associated with arterial stiffness despite dairy consumption being inversely associated with cfPWV[19]. In addition, baseline CCA-IMT was not associated with any serum lipid species. In the present study, changes in serum lipid species were associated with the change in central blood pressure (LPC 14:0, LPC 15:0, LPC 16:1, PC 29:0, PC 30:0, PC 31:0) and the change in CCA-IMT (CE 14:0). In addition, baseline concentration of PC 34:4, PC 32:2, GM3 16:0, GM3 18:0 and total GM3 were associated with the change in CCA-IMT such that higher baseline levels were associated with greater CCA-IMT regression. GM3 accumulation has been observed in intimal atherosclerotic lesions in the carotid artery and aorta[34] and serum GM3 has been identified as a potential marker of early atherosclerosis[35]. Therefore this finding suggests that those with greater atherosclerotic burden at baseline had greater CCA-IMT regression which is consistent with our previous finding[22].

This study shows that when dairy associated serum lipids species were reduced at 3-mo there was a reduction in central blood pressure and CCA-IMT. Conversely when there was an increase in these serum lipid species central blood pressure increased. This finding is not consistent with previous research showing an inverse association between dairy consumption and blood pressure[17,36]. Machin *et al*[36] showed that consuming a high dairy diet (non-fat) for 4 wk reduced central blood pressure and cfPWV compared with a high fruit diet. The relationship we observed whereby an increase in LPC 14:0, LPC 15:0 and LPC 16:1 was associated with an increase in central blood pressure may be explained by the proinflammatory and atherogenic properties of non-omega 3 polyunsaturated fatty acid enriched LPC[37]. LPCs act on endothelial cells, smooth muscle cells, monocytes, macrophages and T-cells in a number of ways to inhibit endothelial relaxation and up-regulate production of inflammatory and adhesion molecules[32,38]. Inflammation, measured by CRP, has been positively associated with blood pressure and development of hypertension[39,40], which may explain our findings in the current study. As such, our results may not be due to the fatty acid composition *per se* but fatty acid metabolism

Table 4 Anthropometric measures, blood pressure, biochemistry and vascular measurements at baseline and follow-up

	Baseline	3 mo	*P* value for change
Weight (kg)	96.2 ± 20.6	95.9 ± 20.6	0.40
Peripheral systolic blood pressure (kPa)	16.9 ± 2.0	16.9 ± 1.9	0.75
Peripheral diastolic blood pressure (kPa)	9.4 ± 1.3	9.4 ± 1.3	0.69
Peripheral mean arterial pressure (kPa)	12 ± 1.3	12 ± 1.3	0.69
Peripheral pulse pressure (kPa)	7.4 ± 1.3	7.4 ± 1.7	0.93
Central systolic blood pressure (kPa)	16.8 ± 1.3	16.1 ± 1.9	0.004
Central diastolic blood pressure (kPa)	10.9 ± 1.3	10.5 ± 1.2	0.002
Central mean arterial pressure (kPa)	13.2 ± 1.5	12.8 ± 1.3	0.002
Central pulse pressure (kPa)	5.9 ± 1.7	5.8 ± 1.7	0.09
Central augmented pressure (kPa)	1.2 ± 0.6	1.1 ± 0.8	0.07
Augmentation index (%)	19 ± 7	21 ± 12	0.005
cfPWV (m/s)	9.4 ± 1.7	9.4 ± 1.8	0.63
Pulse transit time (m/s)	60 ± 10	60 ± 9	0.95
CCA-IMT (mm)	0.72 ± 0.12	0.71 ± 0.12[1]	0.002
Total cholesterol (mmol/L)	3.7 ± 1.0	3.7 ± 1.0	0.59
HDL cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.3	0.54
LDL cholesterol (mmol/L)	2.0 ± 0.8	2.0 ± 0.7	0.40
Triglycerides (mmol/L)	1.1 ± 0.9	1.1 ± 0.9	0.53
Glucose (mmol/L)	7.3 ± 2.9	7.3 ± 2.7	0.95
High sensitivity C reactive protein (mg/L)	2.5 ± 2.4	2.4 ± 2.4	0.80

[1]Follow-up was at 12 mo. All values are mean ± SD. Data analysed using paired samples *t*-test. cfPWV: Carotid femoral pulse wave velocity; CCA-IMT: Common carotid artery intima media thickness; HDL: High density lipoprotein; LDL: Low Density lipoprotein.

Table 5 Spearman's correlation between the absolute and percent change in dairy associated lipid species and the absolute and percent change in vascular measures

	Absolute change		Percent change	
	rho value	*P* value	rho value	*P* value
Central systolic blood pressure				
LPC 14:0	0.30	0.007	0.28	0.01
PC 30:0	0.28	0.010	0.31	0.004
PC 31:0	0.20	0.07	0.22	0.046
Central diastolic blood pressure				
LPC 14:0	0.32	0.004	0.32	0.00
LPC 15:0	0.23	0.04	0.22	0.046
LPC 16:1	0.23	0.035	0.21	0.06
PC 29:0	0.28	0.01	0.34	0.002
PC 30:0	0.36	0.001	0.40	0.001
PC 31:0	0.30	0.007	0.31	0.004
Central mean arterial pressure				
LPC 14:0	0.30	0.007	0.29	0.009
PC 29:0	0.21	0.06	0.25	0.025
PC 30:0	0.31	0.005	0.33	0.002
PC 31:0	0.24	0.033	0.24	0.028
Augmentation index				
Nil significant				
cfPWV				
Nil significant				
CCA-IMT				
CE 14:0	0.22	0.02	0.23	0.02

LPC: Lysophosphatidylcholine; PC: Phosphatidylcholine; CE: Cholesterol ester; cfPWV: Carotid femoral pulse wave velocity; CCA-IMT: Common carotid artery intima media thickness.

and resulting lipid species. Previously total phospholipid C 15:0 has been inversely associated with blood pressure and coronary heart disease[41]. Although it remains unclear what the mechanism behind this finding is and this should be explored in the future.

Ruminant fatty acids C 15:0 and C 17:0 do not have the same physiological effects as other saturated fatty acids (although they have not been tested in isolation) and are not associated with increased risk of cardiovascular outcomes and may actually be protective[42,43]. Dairy products also contain many other components such calcium, magnesium, phosphorus, potassium and bioactive peptides that may contribute to the cardio-protective effect of dairy consumption[44-46]. In a four-way cross-over study it was shown that the calcium content of dairy products counteracts the fat content to attenuate the increase in total and LDL cholesterol, without reducing HDL cholesterol[44]. The synergist effect of the different components in dairy products may explain why dairy consumption was not associated with any of the vascular measurements in the current study despite the observed association between the lipid species and these measurements.

Nestel *et al*[21] showed that the phospholipid classes LPC and lysoalkylphosphatidylcholine were associated with measures of insulin sensitivity and insulin resistance. In addition, full fat dairy consumption was associated with phospholipid fatty acids C 15:0, C 16:1 and C 18:1 n-7 but there was no relationship detected between dairy consumption and insulin sensitivity or resistance measures. This is similar to the findings of the present study whereby the change in dairy consumption was not associated with the change in central blood pressure or CCA-IMT, but the change in a number of lipid species was associated with both the change in dairy intake and the change in central blood pressure and CCA-IMT. This may be because lipid analysis provides a more objective measure of intake and is not limited by the measurement error associated with self-reported dietary intake, although it may be due to a lack of statistical power.

Limitations of this study are that it is observational and therefore causation cannot be established. In addition, just over half of the cohort were prescribed lipid lowering medication which may confound the results. Finally, this study comprises a small sample size and findings should be replicated in a larger cohort. We also did not have lipidomic data at 12-mo but we assumed changes early in the observation period would be required in order see changes in IMT at 12-mo.

In conclusion, in this cohort with type 1 and type 2 diabetes a number of serum lipid species that were associated with a change in full fat dairy consumption were also correlated with the 3-mo change in central blood pressure and 12-mo change in CCA-IMT.

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COMMENTS

Background

People with diabetes are at higher risk of cardiovascular disease than the general population. And poor diet is a leading contributor to the development of cardiovascular disease. At present the effect of dairy consumption on vascular health is unclear. In this study the authors aimed to determine lipid species that change in response to a change in dairy consumption. In addition, to investigate whether dairy associated lipid species are correlated with changes in measures of vascular structure and function.

Research frontiers

Measurement of dietary intake is challenging due to inaccurate reporting, which is well documented in people with diabetes. Previously it has been shown that serum lipid species of ruminant origin are correlated with dairy consumption. In this study, the authors investigated the association between dairy intake measured by dietary questionnaire and serum lipid species and vascular health.

Innovations and breakthroughs

These analyses show that a change in the concentration of a number of serum lipid species (LPC 14:0, LPC 15:0, LPC 16:1, PC 29:0 PC 30:0, PC 31:0, CE 14:0), previously shown to be correlated with dairy consumption, were associated with a change in full fat dairy consumption. One or more of these lipid species were positively associated with the 3-mo change in central blood pressure and 12-mo change in common carotid artery intima media thickness (CCA-IMT) in this cohort with type 1 and type 2 diabetes.

Applications

Due to the observational nature of this research these findings are hypothesis generating and should be confirmed in the future.

Terminology

CCA-IMT is visualized using B mode ultrasound. The intima-media complex is the area of tissue starting at the luminal edge of the artery and ending at the boundary between the media and the adventitia. CCA-IMT is a measure of early atherosclerosis. Augmentation index and carotid femoral pulse wave velocity are non-invasive measures of arterial stiffness.

Peer-review

In the manuscript, the authors conducted a subanalysis of a previous randomized trial addressing the role of diary intake on vascular function and blood pressure parameters. They documented a significant relationship between blood pressure and augmentation index and certain lipid parameters. The study is well conducted with a huge number of parameters analysed and the results are intriguing.

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Clinical Trials Study

Nonsurgical periodontal-therapy improves glycosylated hemoglobin levels in pre-diabetic patients with chronic periodontitis

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Abstract

AIM

To evaluate the effect of nonsurgical periodontal therapy on glycosylated haemoglobin levels in pre-diabetic patients with chronic periodontitis (CHP).

METHODS

Sixty pre-diabetic patients with CHP were selected and equally allocated to case and control group. All subjects were evaluated at base line for periodontal parameters (plaque index, oral hygiene index, modified gingival index, probing pocket depth, clinical attachment level) and systemic parameters [glycosylated hemoglobin (HbA1c), fasting lipid profile, and fasting blood glucose]. The case group received non-surgical periodontal therapy. Subjects were re-evaluated for periodontal and systemic parameters after three months.

RESULTS

Both groups were comparable at baseline. Three months after non surgical periodontal therapy (NSPT), there was significant improvement in periodontal parameters in case group. The mean difference in systemic parameters like HbA1c and fasting plasma glucose from baseline to fourth month for case group was 0.22 ± 0.11 and 3.90 ± 8.48 respectively and control group was -0.056 ± 0.10 and -1.66 ± 6.04 respectively, which was significant between case and control group ($P < 0.05$). In the case group there was a significant decrease in HbA1c from baseline to three months following NSPT ($P < 0.05$).

CONCLUSION

This study showed that periodontal inflammation could affect the glycemic control in otherwise systemically healthy individuals. Periodontal therapy improved periodontal health status and decreased glycosylated haemoglobin levels, thus reducing the probability of occurrence of inflammation induced prediabetes in patients with CHP.

Key words: Prediabetes; Chronic periodontitis; Non surgical periodontal therapy

Core tip: A bidirectional link exists between diabetes and periodontitis. Periodontitis may affect glycemic control in otherwise systemically healthy individuals resulting in an elevated glycosylated hemoglobin level. Pre diabetes may be associated with periodontal disease in systemically healthy subjects. This clinical trial evaluated the effect of non-surgical periodontal therapy on glycosylated hemoglobin levels in pre-diabetic patients with chronic periodontitis (CHP). This study showed that periodontal therapy improved periodontal health status and decreased glycosylated hemoglobin levels, thus reducing the probability of occurrence of inflammation induced prediabetes in patients with CHP.

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INTRODUCTION

Periodontal disease is a complex immuno inflammatory disease characterized by the destruction of periodontal ligament and alveolar bone with subsequent clinical attachment loss. The progression and severity of the periodontal destruction depends on the balance between the virulence of microorganism and the host immune response[1]. Pathogenesis of periodontal disease involves cytokines and inflammatory mediators including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, prostaglandin E 2 (PGE2), *etc*., which are capable of acting alone or together to stimulate the destruction of attachment apparatus. Periodontal pocket acts as a portal of entry for these microorganisms and inflammatory mediators into the systemic circulation which could lead to low-grade inflammatory burden. Recent studies have demonstrated that chronic periodontitis (CHP) is a potential risk factor for systemic diseases like coronary heart diseases/ atherosclerosis[2], diabetes mellitus (DM)[3], *etc*.

DM is a group of metabolic disorder characterized by high blood glucose levels resulting from defect in insulin secretion, insulin action or both[4]. The prevalence of DM across the world is 5%[5]. Periodontitis is accepted as one of the complications of DM[6]. Researchers have identified a two way relation that exists between diabetes and periodontal disease. The biological plausibility supporting such a link is based on the fact that pathogenic biofilm associated with periodontal disease induces a chronic systemic inflammation, and thus contributes to cumulative inflammatory burden which can worsen insulin resistance and impair glycemic control in diabetes[3,7].

ADA Standards of Medical Care in Diabetes introduced glycosylated hemoglobin (HbA1c) level as another criterion for diagnosis of diabetes[4]. According to this, the normal value of HbA1c is $\leqslant$ 5.6%. However, values of, HbA1c $\geqslant$ 6.5% is diabetic and those in between 5.7-6.4 is "pre-diabetic" or "at risk of diabetes". Prediabetes is a high-risk state for diabetes that is defined by glycemic variables that are higher than normal, but lower than diabetic thresholds. Prediabetes is associated with the simultaneous presence of insulin resistance and β-cell dysfunction[8]. International Diabetes Federation projects an increase in the prevalence of prediabetes to 471 million globally by 2035[9].

Although many studies[10-12] have examined the severity of periodontal disease in patients with DM, relatively very few studies[13,14] have addressed the association between periodontitis and glycosylated hemoglobin. These studies reported that HbA1c levels were slightly elevated in CHP patients, otherwise systemically healthy and they were in a pre- diabetic stage. So it can be considered that there exists a relation between prediabetes and periodontal inflammation.

Emerging data suggest that non surgical periodontal therapy (NSPT) can reduce the bacterial deposit, cytokine levels and may result in the reduction of HbA1c and improvement in glycemic status in patients with DM and CHP[15]. So it was hypothesized that NSPT in pre-diabetic patients with CHP would have an effect on their HbA1c level. This study aimed to assess the effect of NSPT on HbA1c level in CHP subjects (otherwise systemically healthy) with HbA1c level in the pre-diabetic range (5.7%-6.4%) and the secondary outcome of this study was to assess the effect of NSPT on serum lipid profile.

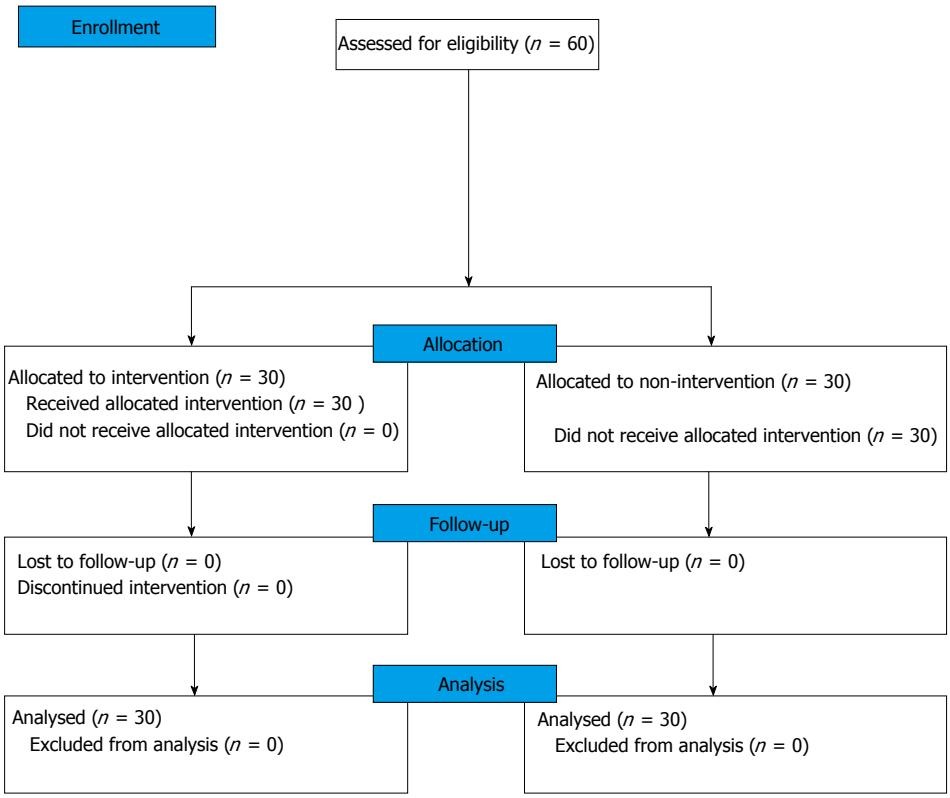

Figure 1 Consort flow diagram.

MATERIALS AND METHODS

Participants

The study was carried out by the Department of Periodontics in association with the Division of Biochemistry, Government-Medical College Calicut. The study subjects were selected from amongst patients who had reported for periodontal treatment and prediabetes was detected from HbA1c.

A total of 60 pre-diabetic patients with CHP (otherwise systemically healthy) reporting to the department of Periodontics were selected as per the inclusion and exclusion criteria. These 60 subjects were equally allocated to case (intervention group) and control group. The inclusion criteria for study subjects were that the patients had to be between the age group of 25 to 55 years, and, have a minimum of 20 teeth present. These patients were otherwise systemically healthy with moderate and severe CHP (CDC criteria[16]) and their HbA1c status in the pre diabetic range (5.7%-6.4%). Patients with systemic diseases/conditions that shortened erythrocyte survival (*e.g.*, hemolytic anemia, chronic kidney disease, pregnancy, *etc.*), acute conditions that contraindicated a periodontal examination, subjects who received systemic antibiotic therapy within the past 6 mo and periodontal therapy in the past one year, and patients who were not willing to sign the informed consent form were excluded from the study. This clinical trial was carried out based on Helsinki Declaration, 2008 modification. The study protocol, consents and all study procedures were approved by the Institutional Ethics Committee, Government Dental College, Calicut, Kerala, India. It was recorded under the clinical trial registry of India (registration No. CTRI/2014/09/004952).

Study design

This clinical trial was a non randomized interventional study of ten months from October 2014 to August 2015 which included steps from enrolment to data analysis. Duration between intervention and re-evaluation was three months (Figure 1). A total of sixty subjects were allocated into the case and control group with thirty subjects in each group. Subjects were assessed by a questionnaire regarding their medical, dental and social history, age, gender, family income, education, occupation and oral hygiene practices.

Oral and periodontal examinations included an assessment of plaque index (PI), calculus index (CI), modified gingival index (MGI), percentage of sites with bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL). Systemic and biochemical parameters included, glycosylated hemoglobin (HbA1c), fasting plasma glucose (FBG), total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL). HbA1c level assay was done by ion exchange high performance liquid chromatography (HPLC). Bio-Rad (D-10) Dual Program kit was used which was NGSP certified and standardized to the DCCT assay. All periodontal and systemic parameters were assessed at

Table 1 Sociodemographic characteristics of case and control group

Sociodemographic character		Case	Control	*P* value
Age (mean ± SD)		41.33 ± 6.63	41.93 ± 5.77	0.710
Gender (% within the group)	Male	26.7	40	0.412
	Female	73.3	60	
Socioeconomic status (% within the group)	APL	73.3	50	0.110
	BPL	26.7	50	

APL: Above poverty line; BPL: Below poverty line.

baseline by a single trained examiner (MS).

Periodontal treatment (intervention)

Oral hygiene instruction was given to subjects in the case group which included demonstration of proper brushing technique and usage of inter proximal cleansing measures. A 10-mL chlorhexidine mouthwash (0.12%) was prescribed for use twice daily for three months. One week prior to periodontal therapy, teeth with poor prognosis were extracted. NSPT (jaw quadrant-wise manner)was done by a single trained investigator (MS) at a series of appointments over a period of two weeks. It included supragingival and subgingival scaling, root planing, and antimicrobial therapy (Cap Amoxycillin 500 mg tid for 5 d). No periodontal treatment was received by control group during the intervention period (3 mo). After the intervention period (3 mo), the control group received non-surgical and supportive periodontal therapy. CHP is not a life-threatening disease. In severe periodontitis, the rate of progression of periodontal destruction is only 0.1 mm per year and by delaying the treatment for 3 mo, the maximum destruction that could occur may be very small (0.025 mm loss) which has no impact on the patient[17].

Re-evaluation

All periodontal and systemic parameters were recorded at the fourth month after NSPT for both case and control group by the same single trained examiner (MS). After the study period all study subjects were advised diet and life style modifications and follow ups.

Statistical analysis

Mean (± SD) and frequency were calculated for quantitative and qualitative variables respectively. Independent *t* test was done to analyse the quantitative variables between groups at baseline and at the time of re-evaluation. χ^2 test was performed to compare characteristics like gender and socioeconomic status at baseline. Mann Whitney *U* test was used to compare modified gingival index, plaque index and calculus index between case and control group at base line. Quantitative variables between baseline and at the time of re-evaluation were analyzed by paired *t* test.

RESULTS

The mean age of case group and control group was 41.33 ± 6.63 and 41.93 ± 5.77 years respectively and was comparable between the groups. There was no significant difference in gender distribution and socioeconomic status between groups ($P > 0.05$) (Table 1). All periodontal and systemic parameters were comparable at baseline ($P > 0.05$) (Tables 2 and 3).

In the case group, the changes in periodontal indices (MGI, PI and CI) from baseline to re-evaluation in the fourth month after NSPT were significant ($P < 0.05$) (Table 4). The percentage of sites with bleeding on probing in case group in the fourth month after NSPT was significantly reduced. In case group at baseline the mean PPD and CAL, was 3.27 ± 0.29, 2.95 ± 0.49 respectively and in the fourth month re-evaluation after NSPT the mean PPD was 2.70 ± 0.24 and mean CAL was 2.48 ± 0.46. The mean changes in PPD and CAL from baseline to re-evaluation was statistically significant ($P < 0.05$) (Table 4). In the case group after NSPT, a significant decrease in PPD and an increase in CAL was observed. Paired *t* test was performed to analyze the mean changes in FBS and HbA1c from baseline to the fourth month in the intervention group. A significant improvement in FBG and HbA1c was observed after NSPT and there was a decrease in mean TC, TG and LDL (Table 5).

The mean changes in periodontal variables from baseline to the fourth month re-evaluation was analyzed between the case and control group subjects. The mean difference in the intervention group for MGI, PI and CI was 1.13 ± 0.32, 0.75 ± 0.21 and 1.73 ± 0.43 respectively and for the control group was -0.006 ± 0.02, -0.003 ± 0.014 and -0.017 ± 0.05 respectively, which was statistically significant ($P < 0.05$). Negative values indicated worsening of periodontal parameters at re-evaluation. The percentage of sites with BOP, percentage of sites with PPD $\leqslant$ 3 mm, PPD4-6 mm, PPD $\geqslant$ 7 mm and percentage of site with CAL 1-2 mm, CAL 3-4 mm, CAL > 5 mm observed significant differences between groups. The mean change in PPD from baseline to fourth month re-evaluation for case and control groups was 0.56 ± 0.16 and -0.01 ± 0.006 and the mean difference in CAL for intervention group and control group was 0.47 ± 0.19 and -0.001 ± 0.019. The mean changes in PPD and CAL were significant between groups, depicting a significant decrease in PPD and gain in CAL for intervention group in the fourth month following NSPT. In control group there was an increase in PPD and an increase in loss of clinical attachment in the fourth month

Table 2 Baseline periodontal parameters of case and control group (mean ± SD)

Parameters	Mean ± SD Case group	Mean ± SD Control group	*P* value
MGI	1.55 ± 0.37	1.53 ± 0.28	0.723
PI	1.05 ± 0.24	1.03 ± 0.23	0.871
CI	1.95 ± 0.45	2.11 ± 0.48	0.173
BOP (%)	60.37 ± 13.66	64.87 ± 11.61	0.175
PPD (mm)	3.27 ± 0.29	3.24 ± 0.27	0.694
Sites with PPD ⩽ 3 mm (%)	60.86 ± 12.22	64.85 ± 8.24	0.143
Sites with PPD 4-6 mm (%)	37.03 ± 11.05	34.05 ± 8.06	0.238
Sites with PPD ⩾ 7 mm (%)	1.75 ± 2.28	1.01 ± 1.46	0.142
CAL (mm)	2.95 ± 0.49	3.03 ± 0.59	0.567
Sites with CAL 1-2 mm (%)	40.58 ± 11.72	41.16 ± 17.16	0.879
Sites with CAL 3-4 mm (%)	42.79 ± 7.76	41.41 ± 10.77	0.573
Sites with CAL ⩾ 5 mm (%)	15.81 ± 10.60	17.67 ± 13.32	0.552

MGI: Modified gingival index; PI: Plaque index; CI: Calculus index; BOP: Bleeding on probing; PPD: Probing pocket depth; CAL: Clinical attachment level.

Table 3 Baseline systemic parameters of case and control group (mean ± SD)

Parameters	Mean ± SD Case group	Mean ± SD Control group	*P* value
FBG (mg/dL)	107.53 ± 9.44	103.17 ± 7.84	0.060
HbA1c (%)	5.96 ± 0.17	5.97 ± 0.19	0.836
TC (mg%)	206.80 ± 38.60	191.83 ± 26.15	0.084
TG (mg%)	130 ± 51.12	129.67 ± 38.98	0.977
HDL (mg%)	44 ± 9.40	43.70 ± 4.23	0.597
LDL (mg%)	135.53 ± 35.45	122.30 ± 23.33	0.093
VLDL (mg%)	25.90 ± 10.26	25.83 ± 7.85	0.970

FBG: Fasting blood glucose; HBA1c: Glycosylated hemoglobin; TC: Total cholesterol; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein.

following NSPT (Table 6).

The mean change in FBG and HbA1c from baseline to the fourth month after NSPT for the case group was 3.90 ± 8.48 and 0.22 ± 0.11 and the control was -1.66 ± 6.04 and -0.056 ± 0.10 which was significant ($P < 0.05$). In the intervention group there was a significant decrease in HbA1c from baseline to three months following NSPT, indicating that NSPT improved glycemic status in these subjects. The mean changes in TC and LDL between groups were significant, whereas the mean differences in TG, VLDL and HDL were not significant ($P > 0.05$) (Table 7).

DISCUSSION

In periodontal disease, cytokines and inflammatory mediators stimulate attachment apparatus destruction *via* tissue-derived matrix metalloproteinases[18]. It has been suggested that proinflammatory cytokines, such as IL-1β and TNF-α, produced as a systemic response to periodontal infection, are responsible for insulin resistance. Taylor *et al*[19] have reported that severe periodontal infection may affect glycemic control in diabetic patients. Wolff *et al*[13] have observed significantly

Table 4 Change in periodontal parameters of case group from base line to fourth month after non surgical periodontal therapy

Parameters	Baseline	At fourth month	*P* value
MGI	1.55 ± 0.37	0.42 ± 0.09	0.000[a]
PI	1.05 ± 0.24	0.30 ± 0.08	0.000[a]
CI	1.95 ± 0.45	0.22 ± 0.11	0.000[a]
BOP (%)	60.37 ± 13.66	19.98 ± 4.35	0.000[a]
PPD (mm)	3.27 ± 0.29	2.70 ± 0.24	0.000[a]
Sites with PPD ⩽ 3 mm (%)	60.86 ± 12.22	82.75 ± 10.28	0.000[a]
Sites with PPD 4-6 mm (%)	37.03 ± 11.05	16.73 ± 10.00	0.000[a]
Sites with PPD ⩾ 7 mm (%)	1.75 ± 0.73	0.47 ± 0.29	0.000[a]
CAL(mm)	2.95 ± 0.49	2.48 ± 0.46	0.000[a]
Sites with CAL 1-2 mm (%)	40.58 ± 11.72	56.17 ± 16.73	0.000
Sites with CAL 3-4 mm (%)	42.79 ± 7.76	34.80 ± 13.49	0.003[a]
Sites with CAL ⩾ 5 mm (%)	15.81 ± 10.60	8.65 ± 7.81	0.000[a]

[a]*P* value ⩽ 0.05. MGI: Modified gingival index; PI: Plaque index; CI: Calculus index; BOP: Bleeding on probing; PPD: Probing pocket depth; CAL: Clinical attachment loss.

Table 5 Change in systemic parameters from baseline and to fourth month after non surgical periodontal therapy in case group (mean ± SD)

Parameters	Baseline	At fourth month	*P* value
FBG (mg/dL)	107.53 ± 9.44	103.63 ± 9.48	0.018[a]
HBA1c (%)	5.96 ± 0.19	5.74 ± 0.22	0.000[a]
TC (mg%)	206.80 ± 38.60	202.63 ± 41.30	0.343
TG (mg%)	130.00 ± 51.12	127.30 ± 55.52	0.598
HDL (mg%)	44.70 ± 9.40	45.87 ± 6.71	0.404
LDL (mg%)	135.53 ± 35.45	129.60 ± 33.71	0.112
VLDL (mg%)	25.90 ± 10.26	25.24 ± 11.15	0.512

[a]*P* value ⩽ 0.05. FBG: Fasting blood glucose; HBA1c: Glycosylated hemoglobin; TG: Triglycerides; TC: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein.

higher blood glucose levels in otherwise systemically healthy patients with periodontitis which indicate that these patients have some dysregulation in their glycemic control and they are in a pre-diabetic state.

Our case group received NSPT including systemic antibiotics, mouth rinses, and periodontal and systemic parameters were reevaluated at the fourth month. NSPT is an effective method to remove the calculus and plaque bacteria attached to the root surface. It results in reduction of the gingival inflammation, probing pocket depth, and improvement in clinical attachment level. Although studies have shown that healing may continue for a period of one year following initial therapy, most of the healing is completed within 3 mo[20,21]. Dentinal tubules which open up as a result of scaling and root planning allow invasion of pathogens and can act as a source of re infection with in a period of 3-4 mo[22]. So in this study re-evaluation of periodontal parameters were done at the fourth month after NSPT.

The various indices measured (MGI, PI, CI) for assessing oral hygiene and inflammatory status had significant

Table 6 Mean difference (mean ± SD) in periodontal parameters from baseline to fourth month re-evaluation for case and control group

Parameters	Mean difference in case group	Mean difference in control group	*P* value
MGI	1.13 ± 0.32	-0.006 ± 0.02	0.000[a]
PI	0.75 ± 0.21	-0.0003 ± 0.014	0.000[a]
CI	1.73 ± 0.43	-0.017 ± 0.05	0.000[a]
BOP (%)	40.39 ± 13.48	-0.41 ± 0.77	0.000[a]
PPD (mm)	0.56 ± 0.16	-0.01 ± 0.006	0.000[a]
Sites with PD ⩽ 3 mm (%)	-21.89 ± 10.10	0.08 ± 0.46	0.000[a]
Sites with PD 4-6 mm (%)	20.29 ± 10.10	0.05 ± 0.54	0.000[a]
Sites with PD ⩾ 7 mm (%)	1.28 ± 1.75	-0.054 ± 0.20	0.000[a]
CAL (mm)	0.47 ± 0.19	-0.001 ± 0.019	0.000[a]
Sites with CAL 1-2 mm (%)	-15.59 ± 11.53	0.078 ± 0.42	0.000[a]
Sites with CAL 3-4 mm (%)	7.99 ± 13.25	0.093 ± 0.43	0.000[a]
Sites with CAL ⩾ 5 mm (%)	7.16 ± 4.79	-0.13 ± 0.36	0.000[a]

[a]*P* value ⩽ 0.05. MGI: Modified gingival index; PI: Plaque index; CI: Calculus index; BOP: Bleeding on probing; PPD: Probing pocket depth; CAL: Clinical attachment loss.

Table 7 Mean difference (mean ± SD) in systemic parameters from baseline to fourth month re-evaluation for case and control group

Parameters	Mean difference in case group	Mean difference in control group	*P* value
FBG (mg/dL)	3.90 ± 8.48	-1.66 ± 6.04	0.005[a]
HBA1c (%)	0.22 ± 0.11	-0.056 ± 0.10	0.000[a]
TC (mg%)	4.16 ± 23.66	-6.43 ± 16.38	0.048[a]
TG (mg%)	2.70 ± 27.71	4.50 ± 27.65	0.802
HDL (mg%)	-1.16 ± 7.54	0.00 ± 3.05	0.436
LDL (mg%)	5.93 ± 19.81	-7.16 ± 16.02	0.007[a]
VLDL (mg%)	0.65 ± 5.38	0.96 ± 5.50	0.824

[a]*P* value ⩽ 0.05. FBG: Fasting blood glucose; HBA1c: Glycosylated hemoglobin; TG: Triglycerides; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TC: Total cholesterol.

improvement following NSPT in the intervention group. This was in accordance with earlier studies by da Cruz *et al*[23] in 2008. He opined that NSPT improved plaque score and ginigival score in CHP patients with or without diabetes. For the control group changes in periodontal indices (MGI, PI and CI) from baseline to re-evaluation were not statistically significant but there was an increase in the scores of these indices and worsening of oral hygiene. It implied that NSPT had an important role in periodontal health maintenance of pre-diabetic patients with CHP. There was a significant reduction in the percentage of sites with bleeding upon probing in the intervention group group following NSPT which was in accordance with Tervonen *et al*[24].

The case group showed an improvement in periodontal parameters like reduction in the probing pocket depth, significant improvement in the clinical attachment level, significant reduction in the percentage of sites with PPD 4-6 mm and ⩾ 7 mm and increase in the percentage of site with PPD 1-3 mm. This observation connotes to the fact that, the average pocket depth had reduced to < 3 mm after NSPT and there by the overall surface area of the pocket lining which acts as a reservoir for periodontal pathogen and inflammatory markers had also reduced. This finding was in accordance with previous studies where significant pocket depth reduction had been observed after NSPT in non diabetic and diabetic patients[25,26]. In control group there was an increase in probing pocket depth and clinical attachment level. This may be due to the worsening of oral hygiene status in this group.

In this study the glycemic control of patients was assessed using FBS and HbA1c. HbA1c is a highly specific and convenient alternative to FBS for diabetes screening. HbA1c is preferred because it reflects the glucose level in blood over the preceding three months as glucose is irreversibly bound to haemoglobin. HbA1c assay can avoid the problem of day-to-day variability of glucose values and the need for the patient to fast before blood collection.

In this study the baseline HbA1c level of the case and control group was in the pre-diabetic range of 5.7 to 6.4. Observational studies by Wolff *et al*[13] and Saxena *et al*[14] have reported that non diabetic individuals with periodontitis have higher HbA1c levels. In this study it was noted that there was a significant difference in change in FBG and HbA1c in the case and control group ($P < 0.05$). The mean reduction in HbA1c for the case group after NSPT was 0.22 ± 0.11, while the control group showed a mean increase in HbA1c value of 0.056 ± 0.10 during re-evaluation, implying that non-surgical periodontal therapy improved the glycemic status in pre-diabetic patients with CHP. The mean reduction of 0.22% HbA1c observed in this study cannot be ignored. The Joint European Federation of Periodontology and American Academy of Periodontology workshop in 2013 had concluded that in diabetic patients 0.36% HbA1c reduction obtained following periodontal interventions was equivalent to those achieved by adding a second drug into their pharmacological regimen[27].

In periodontitis, the chronic exposure to subgingival microorganisms leads to systemic inflammation, which in turn is an independent risk factor for insulin resistance and elevated HbA1c levels in DM. Periodontal therapy in diabetes has been noted to result in changes in systemic monocytic gene expression as well as a decrease in systemic inflammation and insulin resistance leading to a reduction in HbA1c levels[28]. Many studies have investigated the effect of nonsurgical periodontal therapy on periodontal clinical parameters, inflammatory markers, HbA1c, and concluded that all parameters had significantly improved after 3 mo[29,30]. Mammen *et al*[31] in 2016 noticed that NSPT reduced insulin resistance and improved insulin sensitivity in diabetic patients with CHP. The improved HbA1c levels observed in the case group could be attributed to the reduction in inflammation achieved by NSPT and the accumulation of inflammation could have compounded for elevated HbA1c level in

control group.

In accordance with our result, Perayil *et al*[26] in 2014, have reported that there was significant reduction in the HbA1c levels after NSPT in non diabetic individuals with periodontitis. The mean reduction in HbA1c levels obtained in the current study is comparable to the reduction reported by them. In this study, adjuvant antibiotics (Amoxycillin) along with NSPT could have contributed to the improved periodontal clinical parameters. Feres *et al*[32] in 2015 have suggested that scaling and root planning alone does not lead to major clinical improvements in the case of advanced disease with deep pockets. Broad spectrum antibiotics like Amoxycillin potentially control the periodontal pathogens present in epithelial cells and connective tissue which potentiate the effect of mechanical debridement leading to rapid reduction of bacterial load in the subgingival space. In contrast to this report Javed *et al*[33] reported that NSPT reduced hyperglycemia and periodontal inflammation irrespective of the use of antibiotics (Doxycycline).

Among the various parameters of lipid profile assessed in our study, the mean TC and LDL levels of the case group was slightly higher than normal. This is in agreement with the study of Lösche *et al*[34] in 2000 who reported a higher lipid profile in patients with moderate periodontal diseases. Pro inflammatory cytokines such as IL-1, IL-6 and TNF-α released during inflammation can have a direct or indirect effect causing enhanced lipogenesis. Contradictory to this Machado *et al*[35] in 2005 have demonstrated no significant relation between lipid levels and intensity of periodontal diseases.

It was observed that there was a decrease in TC, TG, LDL, and VLDL and an increase in HDL among the case group when evaluated in the fourth month after NSPT, These findings corroborate with the study of D'Aiuto *et al*[36] where they noticed a reduction in the lipid profile of patients undergoing NSPT.

Periodontitis induces a state of systemic subclinical inflammation by peridontopathogens and host mediated inflammatory markers. Systemic inflammation act on pancreatic β cells and change the insulin signaling. The present study noted that non-surgical periodontal treatment was related with improved glycemic control in pre-diabetic patients with CHP, which was evaluated by reduction in the HbA1c levels. Reduction in HbA1c levels after periodontal treatment may be associated with a reduction in insulin resistance and an improvement in insulin sensitivity by the reduction in pathogenic bacterial burden and inflammatory cytokines. This result supports the evidence from previous observational studies which indicate that systemically healthy patients with periodontitis have higher glycemic levels than healthy controls, thus further establishing the bidirectional link between periodontitis and glycemic control. It also emphasizes the need to make the public and medical practitioners aware about the importance of maintaining proper oral health for good systemic health.

In this study short term follow-up was done at fourth month. To understand the long term effects of NSPT on glycemic control, re-evaluation period can be extended to 6 mo and 12 mo period. Periodontal parameters like PPD, CAL were measured using a Williams graduated manual periodontal probe and errors during manual recording are possible. Computerized probes can be used for more accurate PPD and CAL measurements. A randomized double blinded multicentric clinical trial with a larger sample size, with longer duration and evaluation of inflammatory markers like IL-1, IL-6, TNF-α and MMPs are needed to confirm the effect of NSPT on HbA1c in pre diabetic patients with CHP.

Based on the results obtained from this comparative clinical trial, we concluded that NSPT was effective in reducing glycosylated hemoglobin level in pre-diabetic subjects with CHP. Even though the HbA1c level was not attained to the normal level ($\leqslant$ 5.6%) NSPT was effective in improving the glycemic control in pre-diabetic subjects with CHP.

COMMENTS

Background

Periodontal disease is a complex immuno inflammatory disease characterized by the destruction of periodontal ligament and alveolar bone with subsequent clinical attachment loss. Pathogenesis of periodontal disease involves the active expression of both cytokines and inflammatory mediators including interleukin (IL)-1, IL-6, tumor necrosis factor-α, prostaglandin E 2, *etc*. which are capable of acting alone or together to stimulate alveolar bone resorption and collagen destruction. Periodontal pocket acts as a portal of entry for these microorganisms and inflammatory mediators into the systemic circulation which could lead to low-grade inflammatory burden. This study evaluated the effect of periodontal therapy on pre diabetic patients with chronic periodontitis.

Research frontiers

Periodontitis is considered the sixth complication of diabetes mellitus and researchers have identified a bidirectional link that exists between diabetes and periodontal disease.

Innovations and breakthroughs

Periodontitis may affect the glycemic control in otherwise systemically healthy individuals resulting in an elevated glycosylated hemoglobin.

Applications

Periodontal interventions improved periodontal status and decreased glycosylated hemoglobin level, thus reducing the probability of occurrence of inflammation induced pre diabetes in patients with chronic periodontitis.

Terminology

Non surgical periodontal therapy (NSPT): An effective type of periodontal treatment modality that includes supragingival and subgingival scaling, root planing, and antimicrobial therapy.

Peer-review

Generally well-written.

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ORIGINAL ARTICLE

Observational Study

Impact of creatinine methodology on glomerular filtration rate estimation in diabetes

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Institutional review board statement: The study was approved by the Ethics Committee of the Merkur University Hospital, Zagreb, Croatia.

Informed consent statement: This study was waived by IRB due to the retrospective nature of the study, with the post-hoc selection of anonymized samples from the routine laboratory visits, according to creatinine and glucose results.

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Abstract

AIM

To evaluate the influence of creatinine methodology on the performance of chronic kidney disease (CKD)-Epidemiology Collaboration Group-calculated estimated glomerular filtration rate (CKD-EPI-eGFR) for CKD diagnosis/staging in a large cohort of diabetic patients.

METHODS

Fasting blood samples were taken from diabetic patients attending our clinic for their regular annual examination, including laboratory measurement of serum creatinine and eGFR.

RESULTS

Our results indicated an overall excellent agreement in CKD staging (kappa = 0.918) between the Jaffé serum creatinine- and enzymatic serum creatinine-based CKD-EPI-eGFR, with 9% of discordant cases. As compared to the enzymatic creatinine, the majority of discordances (8%) were positive, *i.e.*, associated with the more advanced CKD stage re-classification, whereas only 1% of cases were negatively discordant if Jaffé creatinine was used for eGFR calculation. A minor proportion of the discordant cases (3.5%) were re-classified into clinically relevant CKD stage indicating mildly to moderately decreased kidney function (< 60 mL/min per 1.73 m^2). Significant acute and chronic hyperglycaemia, assessed

as plasma glucose and HbA_{1c} levels far above the recommended glycaemic goals, was associated with positively discordant cases. Due to a very low frequency, positive discordance is not likely to present a great burden for the health-care providers, while intensified medical care may actually be beneficial for the small number of discordant patients. On the other hand, a very low proportion of negatively discordant cases (1%) at the 60 mL/min per 1.73 m^2 eGFR level indicate a negligible possibility to miss the CKD diagnosis, which could be the most prominent clinical problem affecting patient care, considering high risk of CKD for adverse patient outcomes.

CONCLUSION

This study indicate that compensated Jaffé creatinine procedure, in spite of the glucose-dependent bias, is not inferior to enzymatic creatinine in CKD diagnosis/staging and therefore may provide a reliable and cost-effective tool for the renal function assessment in diabetic patients.

Key words: Diabetes; Estimated glomerular filtration rate; Chronic kidney disease-Epidemiology Collaboration Group; Creatinine; Enzymatic method; Chronic kidney disease; Impact; Compensated Jaffé method

Core tip: Analytical performance of the serum creatinine assays is the critical determinant of estimated glomerular filtration rate (eGFR) accuracy. The most widely used compensated Jaffé creatinine assay suffers from a non-specific bias from pseudo-creatinine chromogens (glucose, ketones), which is not the case with the costly enzymatic assays. We evaluated the influence of creatinine methodology on the performance of chronic kidney disease (CKD)-Epidemiology-calculated eGFR for CKD diagnosis/staging in diabetic patients. Our results indicate that compensated Jaffé creatinine procedure, in spite of the glucose-dependent bias, is not inferior to enzymatic creatinine in CKD diagnosis/staging and therefore may provide a reliable and cost-effective tool for the renal function assessment in diabetic patients.

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INTRODUCTION

Global prevalence of diabetes mellitus is rising progressively[1]. Chronic morbidity, associated with various debilitating complications, increased risk for adverse health-outcomes and significant impact regarding both the working ability and quality of life identify diabetes as one of the greatest health-care and socio-economic challenges worldwide. Appropriate strategies to tackle diabetes epidemic include education and lifestyle interventions, evidence-based clinical management as well as the screening for and monitoring of diabetes and/or diabetes-related complications using state-of-the art diagnostic tools.

Diabetic kidney disease (DKD) is one of the most prevalent chronic complications of diabetes and the most common single cause of end-stage renal failure[1,2]. It has been amply evidenced that appropriate interventions at an early stage of DKD can be efficient in preventing and/or delaying the progression of kidney disease and improving patient outcomes. Thus, the regular screening for DKD has became one of the cornerstones of diabetes care. Current clinical guidelines recommend at least an annual screening of DKD in patients with type 1 diabetes with a duration above 5 years, in all patients with type 2 diabetes and in all hypertensive diabetic patients[3]. Once detected, DKD is treated according to clinical guidelines and further monitored at regular intervals[2,3]. Two simple laboratory tests are used for both the screening and staging of CKD in diabetes: Urinary albumin excretion (UAE) and serum creatinine-based estimated glomerular filtration rate (SCr-eGFR).

Abnormal UAE has long been identified as a sensitive marker of the glomerular basal membrane damage, which is one of the early pathophysiological events in the development of DKD[4]. However, a significant decline in eGFR is a common finding in a notable proportion of diabetic patients with normal UAE, probably reflecting a diversity in the natural history of DKD[5]. Thus, the pathophysiology of DKD has shifted from the "albuminuric paradigm"[6], and the accumulated evidence implicating the progressive renal function decline as an equally relevant pathway identified reliable and accurate laboratory testing for serum creatinine and SCr-eGFR as a very important issue for the diagnosis, staging and monitoring of CKD in diabetic patients.

SCr has been used as a cost-effective and practical marker of kidney function for decades, despite severe limitations due to both biological and analytical variability[7]. A handful of biological factors such as age, gender, ethnicity and nutritional habits substantially influence serum creatinine levels, while partial tubular reabsorption and secretion of creatinine further compromise its use as the glomerular filtration marker[8,9]. Nevertheless, SCr-based estimation of GFR by the use of appropriate predictive equation remains the recommended surrogate marker for the assessment of kidney function, since the actual measurement of GFR, due to its complexity and high costs, is not available outside the specialized clinical settings. Current guidelines from the Kidney Disease Improving Global Guidelines (KDIGO) CKD Working Group recommend the use of the chronic kidney disease-Epidemiology Collaboration Group (CKD-EPI) equation[2]. CKD-EPI equation offers an improved reproducibility and accuracy at higher GFR levels (> 60 mL/min per 1.73 m^2), which is the most prominent disadvantage of the

previously recommended Modification of Diet in Renal Disease equation[10].

Analytical performance and specificity of SCr assay are critical determinants of the eGFR accuracy[11]. The relationship between SCr and GFR is exponential, therefore, errors in SCr measurements resulting from imprecision and bias could strongly impact eGFR results and result in misclassification of the patients regarding their kidney function[2]. Despite standardization and harmonization by the calibration traceable to isotope-dilution-mass spectrometry (IDMS), the non-specific bias from pseudo-creatinine chromogens (glucose, proteins, ketone bodies) is still affecting the most widely used compensated Jaffé alkaline picrate colorimetric creatinine assay[11,12]. Enzymatic creatinine methods are free from these interferences, but far more expensive and therefore not widely used. High-volume routine enzymatic creatinine testing may introduce a substantial financial challenge for the laboratories, even in the otherwise fairly resourced health-care systems[13]. Several analytical and clinical studies advocated the replacement of the compensated Jaffé with enzymatic creatinine assays in order to improve reliability of the eGFR, especially in the diabetic population, which is expected to have an increased amount of interfering substances in serum. However, recently published risk-analysis study, using both analytical and biological variability criteria, revealed a low risk for misclassification of CKD based on Jaffé-SCr-eGFR results in the general population[13], while the clinical impact in diabetic population remains unclear.

The aim of this study was to evaluate the influence of creatinine methodology on the performance of CKD-EPI-calculated eGFR for CKD evaluation and staging in a large cohort of diabetic patients.

MATERIALS AND METHODS

Fasting blood samples were taken from diabetic patients attending our clinic for their regular annual examination, including laboratory measurement of SCr and eGFR. Samples from the patients with concomitant infection, limb-amputation and malignancies, as well as the pregnant patients and the patients with severe kidney disease (stage 5, according to KDIGO-2012 classification) were not included in the study. A subset of samples of the patients with severe hyperglycaemia were included in order to evaluate the interference of glucose on the CKD classification across various eGFR categories. Serum creatinine was measured by both IDMS-traceable compensated Jaffé (cJ-SCr) and enzymatic (e-SCr) (Beckman Coulter, Inc., Pasadena, California, United States) procedures with intra-assay imprecision (CV) of 1.58% and 1.39%, respectively. Hexokinase (Beckman Coulter, Inc., Pasadena, California, United States) and NGSP-traceable immunoturbidimetric assays (Tina Quant, Roche, F.Hoffmann-La Roche, Basle, Switzerland) were used for plasma glucose and HbA_{1c} measurement.

Assay-specific SCr-eGFR was estimated by the 4-variable CKD-EPI equation using respective creatinine values[10]. UAE was measured by an automated immunoturbidimetric procedure (Beckman Coulter, Inc., Pasadena, California, United States) in fresh spot urine samples. Urinary creatinine was measured in the same samples and UAE results expressed as the urinary albumin/creatinine ratio.

Staging of albuminuria and CKD, as well as risk assessment for CKD progression was carried out according to KDIGO-2012 criteria (Table 1).

The results were analyzed in the entire population and in sub-groups according to albuminuria (Table 2). Normality of distribution was tested by the Kolmogorov-Smirnov test and the significance of differences between the groups was assessed by the Kruskal-Wallis and Mann-Whitney test, as appropriate. Comparison between the creatinine methods in the study population was tested by Passing-Bablok regression analysis. Specific SCr-eGFR data were compared by Bland Altman analysis, and their agreement regarding clinical CKD staging was evaluated by inter-rater agreement (kappa-analysis). Statistical analyses were performed using MedCalc for Windows, version 9.4.2.0 (MedCalc Software, Ostend, Belgium). $P < 0.05$ was defined as the threshold of significance.

The study was approved by the Merkur University Hospital Ethics Committee. Due to the retrospective nature of the study, with the *post-hoc* selection of anonymized samples from the routine laboratory visits, patient's informed consent was not obtained.

RESULTS

A total of 648 Caucasian diabetic patients (337 males) was included in this study. No gender-related differences were observed in the clinical and biochemical parameters, except significantly lower creatinine levels in females ($P < 0.001$, data not shown). There was a significant increase of SCr and a decrease of eGFR, as measured/estimated by both methods across the categories of albuminuria (Table 2). Fasting plasma glucose was significantly lower in the A3 subgroup only, while HbA_{1c} levels showed no differences regarding albuminuria (Table 2). eGFR and creatinine results did not differ significantly depending on creatinine methodology in either category of albuminuria ($P = 0.228$, 0.2306 and 0.7553 for A1, A2 and A3 category, respectively; Mann-Whitney test).

Passing-Bablok regression analysis revealed a small, but significant constant difference between the enzymatic and compensated Jaffé SCr assays [y = -2.8095 (95%CI: -3.8125 to -1.6066) + 1.0476 × (95%CI: 1.0328-1.0625)] across a wide range of creatinine values (Figure 1). This was accompanied by a minor, but significant creatinine assay-dependent difference in SCr-eGFR values [Bland Altman: y = 1.5154 (95%CI: 1.1635-1.8674; lower limit: -7.4276; upper limit: 10.4585) $P < 0.001$] (Figure 2). The severity of both acute and chronic hyperglycaemia was identified as the significant predictor of between-method SCr-eGFR bias (Spearman's rho = -0.363 and -0.369 for fasting plasma glucose and HbA_{1c}, respectively, $P < 0.001$).

Table 1 Kidney Disease Improving Global Guidelines-2012 Prognostic Categories of Chronic Kidney Disease according to estimated glomerular filtration rate and albuminuria (adapted from the Reference 2)

		Albuminuria categories [albumin/creatinine (mg/mmol)]		
		A1	A2	A3
		< 3	3-30	⩾ 30
		Normal to mildly increased	Moderately increased	Severely increased
eGFR categories (mL/min per 1.73 m^2)	G1, ⩾ 90 Normal/high	Low risk	Moderately increased risk	High risk
	G2, 60-90 Mildly decreased	Low risk	Moderately increased risk	High risk
	G3a, 45-59 Mildly to moderately decreased	Moderately increased risk	High risk	Very high risk
	G3b, 30-44 Moderately to severely decreased	High risk	Very high risk	Very high risk
	G4, 15-29 Severely decreased	Very high risk	Very high risk	Very high risk
	G5, < 15 Kidney failure	Very high risk	Very high risk	Very high risk

eGFR: Estimated glomerular filtration rate.

Table 2 Clinical characteristics of the study subjects across the Kidney Disease Improving Global Guidelines-2012 categories of albuminuria

	Category of Albuminuria		
	A1	A2	A3
N (M/F)	372 (212/198)	166 (87/79)	72 (38/34)
Age (yr)	63 (19-88)	68 (18-88)	60[a,b] (29-85)
Glucose (mmol/L)	9.0 (8.8-9.3)	9.4 (8.9-10.0)	7.4[a,b] (5.7-8.9)
HbA_{1c} (%)	7.4 (7.3-7.6)	7.5 (7.3-7.7)	7.8 (7.4-8.5)
HbA_{1c} (mmol/mol)	59 (57-61)	59 (57-61)	62 (58-71)
e-SCr (μmol/L)	69[b,d] (66-72)	75[a,d] (72-77)	100[a,b] (88-137)
cJ-SCr (μmol/mol)	70[b,d] (67-72)	77[a,d] (72-81)	108[a,b] (87-140)
e-SCr-eGFR (mL/min per 1.73 m^2)	91[b,d] (88-93)	85[a,d] (80-88)	60[a,b] (39-77)
cJ-SCr-eGFR (mL/min per 1.73 m^2)	90[b,d] (87-92)	83[a,d] (76-86)	55[a,b] (39-73)

Age is expressed as median (range) and other variables as median (95%CI of median). [a]$P < 0.001$ *vs* A1; [b]$P < 0.001$ *vs* A2; [d]$P < 0.001$ *vs* A3. HbA_{1c}: Glycosylated hemoglobin; SCr: Serum creatinine; eGFR: Estimated glomerular filtration rate; e: Enzymatic; cJ: Compensated Jaffé; M: Male; F: Female.

Table 3 Reclassification of the estimated glomerular filtration rate based chronic kidney disease stage according to enzymatic and compensated Jaffé creatinine values

		e-SCr-eGFR-based CKD stage					
		1	2	3A	3B	4	Total
cJ-SCr-eGFR-based CKD stage	1	272[1]	5[2]	0[1]	0[1]	0[1]	277 (42.7)
	2	25[3]	206[1]	2[2]	0[1]	0[1]	233 (36)
	3a	0[1]	23[3]	54[1]	0[1]	0[1]	77 (11.9)
	3b	0[1]	0[1]	5[3]	28[1]	1[2]	34 (5.2)
	4	0[1]	0[1]	0[1]	1[3]	26[1]	27 (4.2)
	Total	297 (45.8)	234 (36.1)	61 (9.4)	29 (4.5)	27 (4.2)	648

[1]Concordant; [2]Negatively discordant; [3]Positively discordant cases. CKD: Chronic kidney disease; cJ-SCr-eGFR: Compensated Jaffé serum creatinine-based estimated glomerular filtration rate.

Inter-rater agreement analysis showed an excellent agreement (weighted kappa = 0.918; 95%CI: 0.894-0.94) between the method-specific SCr-eGFRs when classifying subjects into KDIGO-2012 CKD-stages. However, some cases were classified differently between CKD stages depending on the creatinine method used for eGFR calculation (Table 3). Compared to e-SCr-eGFR-based CKD classification, 58/648 (9%) patients were re-classified into a different CKD stage when cJ-SCr-based eGFR was used. The majority of these (54/648; 8%) were re-classified into a more advanced stage of CKD (positive discordance), with 23 (3.5%) cases re-classified into the clinically significant eGFR category indicating mildly to moderately decreased kidney function (< 60 mL/min per 1.72 m^2). Among these, 7 cases (1%) had A1 stage of albuminuria, whereas the rest of clinically significant positive discordant cases had more advanced stages of albuminuria. On the other hand, 8/648 (1%)

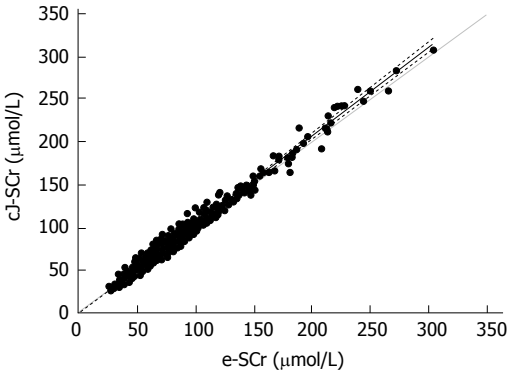

Figure 1 Passing-Bablok regression analysis of the agreement between the enzymatic serum creatinine and compensated Jaffé serum creatinine serum creatinine results in diabetic subjects. e-SCr: Enzymatic serum creatinine; cJ-SCr-eGFR: Compensated Jaffé serum creatinine.

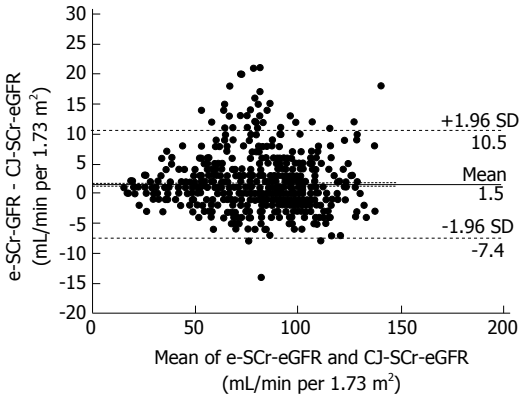

Figure 2 Bland Altman analysis of the agreement between the estimated glomerular filtration rate calculated by using Chronic Kidney Disease-Epidemiology equation with enzymatic serum creatinine-based estimated glomerular filtration rate and compensated Jaffé serum creatinine-based estimated glomerular filtration rate serum creatinine results in diabetic subjects. e-SCr-GFR: Enzymatic serum creatinine-based glomerular filtration rate; cJ-SCr-eGFR: Compensated Jaffé serum creatinine-based estimated glomerular filtration rate.

of patients were re-classified into a less-advanced CKD stage when compensated Jaffé-SCr-eGFR was used (negative discordance), with only 2 cases being re-classified between the 3A and 2 eGFR categories. A1 and A2 stage of albuminuria was detected in each one of these two cases.

There was a significant difference in fasting plasma glucose values regarding concordance of CKD staging, with higher glucose values for positive- and lower glucose values for negative discordant subjects, in comparison to concordant sub-group (11.2 ± 4.3 *vs* 7.5 ± 1.8 *vs* 8.9 ± 2.1 mmol/L, $P < 0.001$). HbA_{1c}, indicating a chronic level of hyperglycaemia, showed an identical pattern (8.4 ± 2.3% ± /69 ± 25 mmol/mol *vs* 6.6 ± 0.7%/49 ± 7.3 mmol/mol *vs* 7.8 ± 1.7%/62 ± 19 mmol/mol; $P < 0.01$). We analyzed the frequency of discordances according to the level of hyperglycaemia, by using the fasting plasma glucose cut-off of 17.0 mmol/L, which was reported to significantly influence SCr results obtained by the colorimetric Jaffé procedure[7]. Positively discordant results were more prevalent in the sub-group of patients with fasting plasma glucose above (n = 59), than below (n = 589), 17.0 mmol/L glucose cut-off (20% *vs* 8%, $\chi^2 = 11.968$, $P = 0.0025$). However, in general, patients with eGFR < 60 mL/min per 1.73 m^2 had lower fasting plasma glucose than those with eGFR > 60 mL/min per 1.73 m^2 (7.3 ± 1.9 mmol/L *vs* 9.2 ± 2.0 mmol/L, $P < 0.0001$).

DISCUSSION

In this study, we attempted to evaluate the influence of creatinine methodology on the performance of CKD-EPI-calculated eGFR for CKD staging in a large cohort of diabetic patients. Our results indicate an overall excellent agreement in CKD staging (kappa = 0.918) between the Jaffé serum creatinine- and enzymatic serum creatinine-based CKD-EPI-eGFR, with 9% of discordant cases. As compared to the enzymatic creatinine, the majority of discordances (8%) were positive, *i.e.,* associated with the more advanced CKD stage re-classification, whereas only 1% of cases were negatively discordant if Jaffé creatinine was used for eGFR calculation. Plasma glucose was identified as a significant determinant of between-method bias.

Kidney function is rather uniquely affected by diabetes. Elevated GFR, known as hyperfiltration, is a common finding in new-onset diabetes, probably as a consequence of hyperglycaemia and related metabolic and endocrine disturbances. Hyperfiltration, being considered as an early sign of DKD[14], is declining with the progression of diabetes and the intensive diabetes treatment was found to be effective in reducing the risk for the progression to DKD by delaying the GFR decline in both type 1 and type 2 diabetes[15,16]. Thus, specific features of DKD in diabetes indicate the need for an accurate and reliable method for GFR estimation over the wide range of GFR. Our previous study showed that CKD-EPI-eGFR, with improved accuracy in the GFR range above 60 mL/min per 1.73 m^2, represented a superior surrogate marker of GFR in diabetic patients, particularly those with normoalbuminuria and hyperfiltration implicating its use as a reliable screening tool for an early renal impairment in diabetes[17]. Either compensated Jaffé or enzymatic creatinine assay, traceable to the reference IDMS procedure, is needed for eGFR-CKD-EPI calculation[2].

Analytical interference of the glucose and other reducing substances in the alkaline picrate Jaffé creatinine assay has long been identified[7]. Several method improvements, including modified spectral kinetics and standardization to the IDMS reference procedure with a mathematical adjustment of results to compensate for interferences (compensated Jaffé assays), have remarkably improved the accuracy of the method. Nevertheless, the non-specificity remained a matter of concern in selected patient subpopulations, such as subjects with diabetes[8]. Enzymatic creatinine assays offer improved specificity and several authors argued that

Jaffé method should be entirely abandoned, particularly in the diabetic population. It was reported that CKD-EPI-eGFR showed better concordance to the measured GFR, empowering further the enzymatic method as a method of choice for serum creatinine measurement in diabetic patients[15]. However, evidence supporting this proposition is based either on cross-sectional method-comparison studies including a limited number of patients, or simulation studies using analytical bias extracted from inter-laboratory comparisons, with no data regarding the clinical outcome-associated risk[18-21]. Our results demonstrate a minor, but significant glucose-dependent positive bias between the serum creatinine levels measured by compensated Jaffé and enzymatic procedure, with a mirroring effect regarding respective eGFRs, but the key question of this study was the clinical relevance of the observed difference.

State-of-the-art strategies for laboratory test evaluation implicate not only analytical, but also clinical performance together with clinical- and cost-effectiveness as essential interactive components of the overall diagnostic test utility assessment[22]. In a recently published outcome-based study, Schmidt *et al*[13] reported on a very low risk for patient outcomes due to the miss-classification of CKD stages with Jaffé creatinine assay in the general population. Our study reveals that most of the discordant cases in diabetic subjects were positive, *i.e.,* Jaffé method was likely to classify 8% of the patients into a more advanced CKD stage than the enzymatic method. Among these, 23/648 cases were classified into the clinically significant stage 3A, while only 7 positively discordant cases with normal UAE (1%) were re-classified between the low4 and moderately increased risk for the CKD progression, according to KDIGO guidelines (Table 1). The frequency of positively discordant cases was 2.5 times greater in the sub-group of patients with plasma glucose above the 17.7 mmol/L, previously reported as a threshold for a significant analytical interference[7]. It is important to emphasize that both fasting plasma glucose and HbA_{1c} in positively discordant sub-group was far above the glycaemic recommendations for adults with diabetes, requiring immediate interventions to reduce the hyperglycaemia regardless of the kidney function[3], which is known to be afflicted by the renal hypoperfusion in acute hyperglycaemic episodes[23,24].

Apart from assessing frequency, our main goal was to evaluate the clinical consequences of the discordant Jaffé creatinine-dependent CKD staging. In general, clinical guidelines recommend the optimization of glycaemic control and blood pressure as the treatment strategy to prevent and/or delay the development/progression of CKD in diabetic patients[3]. Patients with stage 3a CKD (eGFR range 45-60 mL/min per 1.73 m^2) should be referred to a nephrologist for further evaluation, their diet and medication adjusted and eGFR monitoring intensified to twice a year. The more advanced stages of CKD require further specialized care and intensified monitoring, and metformin should be discontinued in patients with stage 3b CKD (30-45 mL/min per 1.73 m^2). However, KDIGO-2012 guidelines recommend repeated eGFR measurement within 3 mo for all subjects with eGFR < 60 mL/min per 1.73 m^2, in order to confirm the classification[2]. Considering the recommended diabetes guidelines, our positively discordant group at the 60 mL/min per 1.73 m^2 eGFR level (3.5%) would not experience any harm from being classified into more advanced CKD stage according to the Jaffé creatinine-based eGFR. On the contrary, clinical intervention to improve glycaemic control was identified as being immediately needed for all these patients, and, if KDIGO-2012-recommended confirmatory eGFR was to be done after 3 mo, presumably reached glycaemic goals would allow more accurate CKD classification not only by the Jaffé method. Limited design of our study did not allow the insight into actual follow-up data, but the well-established ameliorating effect of improved glycaemic control on renal function is likely to elicit the consequent beneficial effect on eGFR *via* biological mechanism(s)[23], regardless of the creatinine method used. Furthermore, the majority of the positively discordant cases had A2 and A3 stages of albuminuria, confirming an increased or high risk of CKD progression in these patients[2].

Significant improvements in CKD screening strategies were enabled by creatinine standardization and automated eGFR reporting by clinical laboratories[11,12]. However, recommended practice is still not implemented in many clinical settings, indicating a lack of appropriate clinical care with significant medical and financial consequences[25]. One of the reasons for the reluctance to implement the guidelines is the concern of increased costs of specific creatinine testing. Our results show that low-cost Jaffé creatinine assay can be safely used for the CKD screening in patients with diabetes, despite confirmed positive analytical bias in comparison to enzymatic assay, provided that compensated Jaffé assay, traceable to the reference IDMS procedure, and CKD-EPI-calculated eGFR are used. The frequency of positively discordant CKD classification is very low and associated with severe hyperglycaemia, where the appropriate interventions to attain glycaemic goals are warranted regardless of CKD. Concomitant presence of increased albuminuria in the majority of discordant cases further diminishes the clinical practice consequences, which may, in turn, be beneficial for the patients in terms of increasing frequency of eGFR monitoring and intensifying efforts to control hyperglycaemia and hypertension. Due to a very low frequency, positive discordance is not likely to present a great burden for the health-care providers. On the other hand, a very low proportion of negatively discordant cases (1%) at the 60 mL/min per 1.73 m^2 eGFR level indicate a negligible possibility to miss the CKD diagnosis, which could be the most prominent clinical problem affecting patient outcomes. Namely, CKD, being a major risk factor for cardiovascular disease and overall mortality, should be detected as early as possible, since appropriate interventions can substantially reduce risks and improve patient outcomes[2].

Limitations of our study include cross-sectional design

and singular ethnicity. A large cohort of diabetic patients stratified according to albuminuria and degree of hyperglycaemia, as well as clinical outcome-based, rather than method-comparison-based approach can be regarded as the study advantages.

In conclusion, results from our study indicate that compensated Jaffé creatinine procedure, in spite of the glucose-dependent bias, is not inferior to enzymatic creatinine in CKD diagnosis/staging and therefore may provide a reliable and cost-effective tool for the renal function assessment in diabetic patients.

COMMENTS

Background

Diabetic kidney disease (DKD) is one of the most prevalent chronic complications of diabetes and the most common single cause of end-stage renal failure. Current clinical guidelines recommend regular screening for and staging of DKD by the laboratory assessement of both urinary albumin excretion rate and estimation of glomerular filtration rate from serum creatinine (SCr-eGFR).

Research frontiers

Analytical performance of the serum creatinine assays is the critical determinant of eGFR accuracy. The most widely used compensated Jaffé creatinine assay suffers from a non-specific bias from pseudo-creatinine chromogens (glucose, ketones), which is not the case with the costly enzymatic assays. Several studies advocated the replacement of the compensated Jaffé with enzymatic creatinine assays in order to improve reliability of the eGFR, especially in the diabetic population, which is expected to have an increased amount of interfering substances in serum. However, recently published risk-analysis study, using both analytical and biological variability criteria, revealed a low risk for misclassification of CKD based on Jaffé-SCr-eGFR results in the general population, while the clinical impact in the diabetic population remains unclear.

Innovations and breakthroughs

This study evaluated the influence of creatinine methodology on the performance of CKD-EPI-calculated eGFR for CKD diagnosis/staging in diabetic patients. The results indicate that compensated Jaffé creatinine procedure, in spite of the glucose-dependent bias, is not inferior to enzymatic creatinine in CKD diagnosis/staging and therefore may provide a reliable and cost-effective tool for the renal function assessment in diabetic patients.

Applications

Significant improvements in CKD screening strategies were enabled in the last decade by creatinine standardization and automated eGFR reporting by clinical laboratories. However, recommended practice is still not implemented in many clinical settings, indicating a lack of appropriate clinical care with significant medical and financial consequences. One of the reasons for the reluctance to implement the guidelines is the concern of increased costs of specific creatinine testing. This results show that low-cost Jaffé creatinine assay can be safely used for the CKD screening in patients with diabetes, despite confirmed positive analytical bias in comparison to enzymatic assay, provided that compensated Jaffé assays, traceable to the reference IDMS procedure, and CKD-EPI-calculated eGFR are used. This finding is particularly valuable for the primary health-care facilities, since available and cost-effective screening for DKD may substantially improve patient outcomes and reduce overall costs associated with more advanced complications of diabetes.

Peer-review

The paper is far from being a useful tool for the clinical management of CKD patients, it shows an interesting new approach to be validated in a prospective way and bigger sample size in order to clarify the potential use in specific clinical situations of CKD patients.

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FIELD OF VISION

Statins redux: A re-assessment of how statins lower plasma cholesterol

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Abstract

Obesity associated dyslipidemia and its negative effects on the heart and blood vessels have emerged as a major healthcare challenge around the globe. The use of statins, potent inhibitors of hydroxyl-methyl glutaryl (HMG) Co-A reductase, a rate-limiting enzyme in cholesterol biosynthesis, has significantly reduced the rates of cardiovascular and general mortality in patients with coronary artery disease. How statins lower plasma cholesterol levels presents a mechanistic conundrum since persistent exposure to these drugs *in vitro* or *in vivo* is known to induce overexpression of the HMG Co-A reductase gene and protein. In an attempt to solve this mechanistic puzzle, Schonewille *et al*, studied detailed metabolic parameters of cholesterol synthesis, inter-organ flux and excretion in mice treated with 3 common statins, rosuvastatin, atorvastatin or lovastatin, each with its unique pharmacokinetics. From the measurements of the rates of heavy water (D_2O) and [^{13}C]-acetate incorporation into lipids, the authors calculated the rates of whole body and organ-specific cholesterol synthesis in control and statin-treated mice. These analyses revealed dramatic enhancement in the rates of hepatic cholesterol biosynthesis in statin-treated mice that concomitantly elicited lower levels of cholesterol in their plasma. The authors have provided strong evidence to indicate that statin treatment in mice led to induction of compensatory metabolic pathways that apparently mitigated an excessive accumulation of cholesterol in the body. It was noted however that changes in cholesterol metabolism induced by 3 statins were not identical. While sustained delivery of all 3 statins led to enhanced rates of biliary excretion of cholesterol and its fecal elimination, only atorvastatin treated mice elicited enhanced trans-intestinal cholesterol excretion. Thus, blockade of HMGCR by statins in mice was associated with profound metabolic adaptations that reset their cholesterol homeostasis. The findings of Schonewille *et al*, deserve to be corroborated and extended in patients in order to more effectively utilize these important cholesterol-lowering drugs in the clinic.

Key words: Statins; Atorvastatin; Lovastatin; Rosuvastatin; Cholesterol synthesis; Hydroxyl-methyl glutaryl-CoA reductase

Core tip: Schonewille *et al*, comprehensively studied cholesterol metabolism (*de-novo* synthesis of cholesterol and its inter-organ flux, and fecal elimination) in mice treated with rosuvastatin, atorvastatin or lovastatin. These analyses revealed that the rates of whole body and organ-specific cholesterol synthesis were boosted by all three statins. Mice treated with statins also elicited enhanced rates of biliary excretion of cholesterol and its fecal elimination. Remarkably, the process of trans-intestinal cholesterol excretion was augmented by only atorvastatin. These data shed mechanistic light on how statin treatment led to organ-specific metabolic adaptations in mice to reset their cholesterol homeostasis.

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INTRODUCTION AND COMMENTARY ON HOT TOPICS

Statins are among the most commonly prescribed drugs across the globe[1,2]. Statins were discovered in the 1970s, as competitive inhibitors of hydroxyl-methyl glutaryl coenzyme-A reductase (HMGCR), the rate limiting enzyme in cholesterol biosynthesis[3]. Soon after their discovery, statins were shown to be relatively safe drugs that caused lowering of serum cholesterol and reduction of fatty streaks in the blood vessels of laboratory animals. The positive outcomes of statin therapy in experimental animals were corroborated and extended to patients in large-scale clinical studies that established that statin treatment was correlated with significantly reduced rates of mortality in patients with atherosclerosis[1,2].

In addition to eliciting salutary effects in the cardiovascular system that includes improved endothelial function and neo-angiogenesis, statins exert pleiotropic actions in various tissues including the central nervous system. For instance, statins appear to exert anti-inflammatory actions in diseases such as rheumatoid arthritis, Alzheimer and Parkinson's disease, and multiple sclerosis[4]. It is worth mentioning however, that statins are far from innocuous since a sub-set of patients taking these drugs develop a range of adverse reactions that include muscle pain or myalgia, and in rare cases, rhabdomyolysis. Additionally, statin therapy has been shown to predispose some patients to develop type 2 diabetes[5].

In spite of decades of research in cultured cells and experimental animals[6], the mechanistic details underlying diverse actions of statins *in vivo* are poorly understood. According to the prevailing mechanism of action, statins competitively block HMGCR enzyme that leads to reduced biosynthesis of cholesterol. Statin-induced depletion of cholesterol in the endoplasmic reticulum (ER) activates the sterol response element binding proteins (SREBP-1 and SREBP-2) cleavage and activation protein, SCAP, that chaperones the full-length transcription factor SREBP2 from ER to the Golgi. The nascent SREBP-2 is sequentially cleaved by regulated intra-membrane proteolysis catalyzed by two proteases, named S1P and S2P. The truncated nSREBP-2 then moves into the nucleus to activate transcription of its downstream targets that include the gene encoding the low-density lipoprotein receptor (LDLR). The LDLRs are the primary regulators of cholesterol homeostasis and their increased abundance on plasma membranes is inversely coupled to levels of LDL-cholesterol in circulation[7,8].

This relatively straightforward relationship between statin-induced blockade of HMGCR and reduced concentration of LDL-cholesterol in plasma fails to reconcile a key observation that persistent exposure to statins leads to enhanced expression of HMG Co-A reductase gene and its enzyme, both in cells in culture and in intact animals[6]. This behavior of statins is not unusual since targeted inhibition of enzymatic pathways often leads the emergence of adaptive mechanisms to cope with such metabolic blockades. The compensatory up regulation of HMGCR expression in patients in response to statins does not hinder their clinical utility since doses of statins are invariably adjusted to achieve the desired range of plasma cholesterol.

Although the pharmacology of statins has been extensively studied, attempts to decipher whether and to what extent these drugs modulate rates of cholesterol biosynthesis and its tissue-specific sequestration and excretion *in vivo* have yielded conflicting results. For example, based on measurements of serum concentration of surrogates of cholesterol (*e.g.*, mevalonic acid, squalene, cholestenol, lathosterol and desmosterol), some investigators have claimed that statin therapy was associated with reduced rates of cholesterol biosynthesis[9,10]. Similarly, the results of direct quantification of cholesterol have also yielded apparently conflicting conclusions. Thus, while one study[11] reported that rates of cholesterol synthesis remained unaltered in patients taking pravastatin or lovastatin, two other reports claimed that statin treatment led to reduced rates of cholesterol biosynthesis[12,13] and, yet another study reached an exactly opposite conclusion[14]. We believe that a clear-cut interpretation of earlier studies has been partially confounded by relative paucity of experiments that were designed to directly measure the rates cholesterol biosynthesis *in vivo*. Additionally, most earlier studies did not rigorously address the relationship between mechanisms involved in the biosynthesis of cholesterol and its inter-organ flux in statin-treated animal or patients. A number of studies have shown the inadequacy of molecular surrogates of cholesterol

to accurately reflect the rates of cholesterol synthesis *in vivo* (discussed in detail in the publication of Schonewille *et al*[15], which is the subject of the current Commentary). Finally, studies to date have not undertaken a sufficiently comprehensive analysis of cholesterol metabolism to specifically reconcile the paradoxical observation that statin therapy was associated with increased expression of HMGCR messenger RNA and protein in the livers of laboratory rodents and patients while concomitantly leading to lower levels of serum cholesterol[16-22].

In a recent paper (Schonewille M, Freark de Boer J, Mele L, Wolters H, Bloks VW, Wolters JC, Kuivenhoven JA, Tietge UJ, Brufau G, Groen AK. Statins increase hepatic cholesterol synthesis and stimulate fecal cholesterol elimination in mice. *J Lipid Res* 2016; 57: 1455-1464), Schonewille *et al*[15], have attempted to re-examine the mechanistic paradox of how statins affect cholesterol metabolism *in vivo*. To achieve this goal, the authors undertook a comprehensive analysis of putative pathways involved in *de novo* biosynthesis of cholesterol and its elimination in mice that were fed rosuvastatin, atorvastatin or lovastatin, mixed with regular rodent chow. The authors chose these 3 common statins because they have unique pharmacokinetics properties. Thus, lovastatin, a pro-drug that needs to be metabolically activated in the enterohepatic circulation, was given to a group of mice; a parallel cohort of mice received rosuvastatin which is a more hydrophilic and liver-selective statin. The third group of mice were fed chow mixed with atorvastatin. Both atorvastatin and lovastatin are more lipophilic and have longer half-lives than rosuvastatin. After 5 d of statin treatment, rates of cholesterol synthesis, sequestration and elimination were assessed in various tissues of control and statin-treated mice.

The authors used two complementary, stable isotope-labeling techniques to compare the rates of whole body and organ-specific cholesterol synthesis. Using the classic method[23], based on the incorporation of deuterium-labeled water (D_2O) into newly synthesized lipids, Schonewille *et al*[15], assessed *de novo* cholesterol synthesis. Since deuterium-enriched body water becomes uniformly distributed in all organs, this technique allows determination of absolute rates of cholesterol synthesis from quantification of deuterium incorporated into lipids. Schonewille *et al*[15], also studied cholesterol metabolism in mice that drank water spiked with 2% [^{13}C]-acetate. Under these conditions, acetate was transported into the liver *via* the portal vein which continuously fluxes acetate from the gastrointestinal (GI) tract to the liver[24]. As documented earlier, the majority of the exogenously supplied [^{13}C]-acetate enters the liver in an intermittent, yet highly efficient manner and gets assimilated into acetyl-CoA and newly synthesized lipids[25,26]. This technique enabled the authors to calculate fractional rates of cholesterol synthesis in control and statin-treated mice. It is noteworthy that although the two techniques measured different facets of *de novo* lipid synthesis, the profiles of absolute rates of cholesterol synthesis derived from incorporation of H^3-enriched drinking water into newly synthesized lipids or fractional rates of hepatic synthesis of cholesterol, as assessed by rates of incorporation of a bolus of exogenous [^{13}C]-acetate, showed similar qualitative and quantitative trends in statin-treated mice that were significantly different from untreated animals.

Based on the kinetics of incorporation of either [^{13}C]-acetate or D_2O water into lipids, the authors surmised that atorvastatin and lovastatin treatments led to a robust increase in *de novo* cholesterol synthesis in the liver. The authors simultaneously measured changes in organ-specific expression of genes relegated to the biosynthesis of cholesterol and molecular pathways that were putatively involved in the accumulation of cholesterol in various tissues. Concomitant quantification of rates of lipid synthesis and gene expression analyses led to three important insights. Thus, Schonewille *et al*[15], observed that: (1) treatment with rosuvastatin, atorvastatin and lovastatin was associated with 6-, 15-, and 11-fold greater expression of hepatic HMGCR protein, respectively; (2) treatment of all 3 statins also led to greater expression of *SREBP-2* gene and some of its downstream targets (*e.g.*, mevalonate kinase, phosphomevalonate kinase, farnesyl-diphosphate farnesyltransferase 1 and squalene epoxidase); and (3) plasma concentrations of 3 cholesterol surrogates (*e.g.*, lathosterol, lanosterol and desmosterol) failed to accurately reflect the rates of cholesterol synthesis in statin-treated mice.

To seek an explanation for the discrepancy between enhanced rates of synthesis of hepatic cholesterol and its lower concentration in the plasma of statin-treated mice, Schonewille *et al*[15], undertook additional experiments aimed at discovering putative pathways involved in inter-organ flux of cholesterol and its elimination. The authors found that hepatic expression of genes encoding LDLR and two ATP-binding cassettes-containing cholesterol transporters, ABCG5 and ABCG8, was greatly enhanced in mice receiving statin treatment. The authors also experimentally probed inter-organ fluxes of cholesterol to determine if excessive cholesterol was removed from the liver and plasma *via* biliary secretion or *via* fecal elimination. Since accumulation of cholesterol in the feces represents 3 potential sources, *i.e.*, unabsorbed dietary cholesterol, cholesterol secreted from the gall bladder, and trans-intestinal cholesterol elimination (TICE), Schonewille *et al*[15], sought evidence for all three sources of cholesterol in the feces of statin-treated mice. Based on these analyses, the authors concluded that while statins did not alter intake of dietary cholesterol or its absorption, statin-mediated inhibition of HMGCR was associated with increased cholesterol in the bile acids; with regard to biliary secretion, lovastatin was found to be particularly potent. It was noted however that the metabolic adaptations induced by 3 statins were not identical. For instance, the atorvastatin-treated mice uniquely elicited increased rates of TICE. These data were consistent with the conclusion that

treatment with either rosuvastatin and lovastatin was associated with robustly increased rates of secretion of hepatobiliary cholesterol and its fecal elimination, atorvastatin treatment also impinged on the mechanism of TICE. Thus, although enhanced cholesterol catabolism might have partially contributed to increased cholesterol synthesis in atorvastatin-treated mice, apparently, this was not the case in mice treated with rosuvastatin or lovastatin. Whether or not statin treatment is associated with enhanced rates of TICE in humans remains to be experimentally established.

Although the question of how statins lower plasma cholesterol has been studied in many laboratories, the report of Schonewille *et al*[15], is remarkable with regard to the comprehensive nature of their experiments aimed at answering this question. Thus, the authors not only explored the molecular mechanisms of biosynthesis of cholesterol (stable isotope-labeling of newly synthesized lipids, gene and protein expression) and its inter-organ flux but also the processes of biliary and fecal elimination of cholesterol in statin-treated mice. A key insight of experiments reported by Schonewille *et al*[15], is that sustained presence of statins *in vivo* leads to significant inter-organ metabolic interactions that profoundly altered cholesterol homeostasis in statin-treated mice. These data also revealed that all statins were not alike with respect to their ability to alter the mechanisms of cholesterol homeostasis.

While the report of Schonewille *et al*[15], contains novel data that bear directly on the actions of statins on whole body and organ-specific cholesterol homeostasis, it raises a number of key mechanistic questions that need to be addressed in follow-up studies. For example, it is not clear from the data of Schonewille *et al*[15], if age of the mice or their gender played a significant role in altered cholesterol metabolism in response to statin treatment. In a similar vein, additional investigations are needed to reveal if effects of statins on the biosynthesis and inter-organ flux of cholesterol are dose-dependent. Finally, future studies need to address if the effects of statins on lipoprotein profiles (HDL *vs* LDL) are species-specific, and if so, what are the underlying molecular mechanisms that explain variable responses of rodents and humans to statin treatment. These caveats notwithstanding, the findings of Schonewille *et al*[15], in mice deserve to be corroborated and extended in other animals, and in patients. Such investigations ought to be launched with an aim to decipher the cellular and molecular mechanisms involved in species-specific differences in statin-induced alterations in the pathways of *de novo* biosynthesis of cholesterol and its inter-organ flux[21,27,28].

CONCLUSION

To sum up, the tantalizing discovery of a novel mechanism of action of statins in mice, reported by Schonewille *et al*[15], has important implication for patients receiving statin therapy. In my opinion, analogous investigations in patients have generally lacked the experimental rigor and scope of the present study, that have perhaps contributed to apparently conflicting data[6,29]. This situation can only be mitigated by more comprehensive studies aimed at directly measuring the rates of *de novo* cholesterol synthesis, inter-organ cholesterol flux and the processes of sequestration and elimination of neutral sterols in humans taking statins. Accomplishing this mission will be predicated on the development of innovative, noninvasive methods to study the mechanisms of cholesterol homeostasis in humans in greater detail. Such analyses are likely to yield new insights that will enable a more judicious use of statins in millions of patients worldwide.

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REVIEW

Diabetes mellitus and stroke: A clinical update

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Abstract

Cardiovascular disease including stroke is a major complication that tremendously increases the morbidity and mortality in patients with diabetes mellitus (DM). DM poses about four times higher risk for stroke. Cardio-metabolic risk factors including obesity, hypertension, and dyslipidaemia often co-exist in patients with DM that add on to stroke risk. Because of the strong association between DM and other stroke risk factors, physicians and diabetologists managing patients should have thorough understanding of these risk factors and management. This review is an evidence-based approach to the epidemiological aspects, pathophysiology, diagnostic work up and management algorithms for patients with diabetes and stroke.

Key words: Diabetes mellitus; Stroke; Metabolic memory; Cardiovascular disease; Glycaemic management

Core tip: With the current global prevalence of more than 415 million, diabetes mellitus (DM) poses very high risk for cardiovascular diseases including stroke. Associated risk factors for stroke such as obesity, hypertension and dyslipidaemia are also high among DM cases especially in those with type 2 diabetes that further increases stroke risk. Thorough understanding of the epidemiology, pathophysiology and management options for patients with DM and co-morbidities is imperative for a rational medical practice among health-care professionals. This review updates a scientific approach to patients with diabetes and stroke.

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INTRODUCTION

Diabetes mellitus is a major risk factor for cardiovascular disease (CVD) including stroke. In 2015, the global prevalence of diabetes was estimated to be 415 million adults, with 12% of global expenditure (US$ 673 billion) on health spent for diabetes care alone[1]. Steady increase in the incidence of type 2 diabetes mellitus (T2DM) related to adverse eating habits, obesity and inadequate physical activity resulted in an exponential rise in diabetes-related cardiovascular morbidity worldwide in recent years. This trend is expected to escalate further with the improvement in life expectancy from advancements in science, technology and health-care resources that resulted in a sharp rise in the proportion of older individuals in the global population with higher prevalence of T2DM and hypertension. World Health Organization's current estimate of 900 million people aged ⩾ 60 years (12% of global population in 2015) is expected to cross 2 billion by 2050 (22% of world population), with 80% of these individuals in the low- and middle-income countries would catalyze the explosiveness of this alarming situation[2].

Being a disease mainly associated with lifestyle, patients with T2DM usually have additional risk factors for stroke such as obesity, hypertension and dyslipidaemia that multiplies the vascular risk in these patients[3]. Type 1 diabetes mellitus (T1DM) also increases the stroke risk although to a lesser degree. Management of diabetes immediately after a stroke and in the long-term follow up period poses significant challenges to clinicians. Inappropriate management of diabetes also increases immediate and long-term morbidity and mortality associated with stroke, and significantly elevates the risk for recurrent strokes[4]. We outline the evidence base for the epidemiological aspects, pathophysiology, diagnostic work up and management algorithms for diabetes and stroke to help clinicians for a rational approach to patients through this comprehensive article.

DIABETES AND STROKE: EPIDEMIOLOGY

Globally, stroke mortality rates have fallen, but stroke incidence and its sequelae have significantly increased over the last three decades[5,6]. Diabetes is a recognized independent risk factor for stroke and is associated with higher morbidity and mortality[7-9]. Table 1 summarises the results of relevant prospective studies demonstrating the relative risk of ischaemic stroke in different diabetes populations worldwide[10-20]. Cardiometabolic risk factors including obesity, hypertension, and dyslipidaemia often co-exist with T2DM and can contribute to the higher reported relative stroke risks when compared to patients with similar risk profile without diabetes[8,21-23].

Table 1 Risk of stroke in diabetes mellitus from different study populations

Study population	Follow-up (yr)	Relative risk (95%CI), gender
Framingham study, 5209 persons, 30-62 years old[10]	20	2.5 (M) 3.6 (F)
Honolulu Heart Program, 7598 men, 45-70 years old[11]	12	2.0 (1.4-3.0)
United States, Nurse Study, 116177 women, 30-55 years old[12]	8	3.0 (1.6-5.7)
Finland, 1298 persons, 65-74 years old[13]	3.5	1.36 (0.44-4.18) M 2.25 (1.65-3.06) F
Sweden, 241000 persons, 35-74 years old[14]	8	4.1 (95%CI: 3.2-5.2) M 5.8 (95%CI: 3.7-6.9) F
United States (ARIC), 15792 persons, 45-64 years old[15]	6-8	2.22 (1.5-3.2)
United Kingdom, 7735 men, 40-59 years old[16]	16.8	2.27 (1.23-4.20)
Renfrew/Paisley, Scotland, 15406 person, 45-64 years old[17]	20	1.52 (0.72-3.21) M 2.83 (1.63-4.90) F
Oldmsted County, Minnesota, 9936 persons, 40-70 years old[18]	15	3.5
United States, Hispanics, 503 persons, 70-90 years old[19]	3.5	3.5 M 5.0 F
Asia, Australia, New Zealand, 161214 persons[20]	5.4	2.09 2.49 Asian population

M: Male; F: Female.

CLINICAL PATTERN OF STROKE IN PATIENTS WITH DIABETES

There are clear differences in stroke patterns between patients with diabetes and those without diabetes. Patients with diabetes have a higher proportion of ischaemic stroke compared to haemorrhagic strokes, and lacunar infarcts (*i.e.*, small 0.2 to 15 mm, non-cortical infarcts) is the most common stroke type. This may be due to the higher prevalence of microvascular disease and the co-existence of hypertension seen in this patient group[24-26]. Table 2 summarises prospective studies highlighting stroke patterns and risk factors identified in patients with diabetes. Prognostic features also differ from normal stroke population as diabetes is associated with an increased risk of subsequent strokes, greater functional disability, longer in-hospital stay, and increased mortality[8,34]. A higher risk of developing stroke-related dementia has also been reported[35].

PATHOPHYSIOLOGICAL CONSIDERATIONS

Hyperglycaemia

It is now evident hyperglycaemia increases oxidative

Table 2 Stroke patterns and risk factors in diabetes *vs* non-diabetes group[1]

Investigators, stroke type	Stroke study population	Stroke patterns diabetes *vs* non-diabetes	Significant stroke risk factors in diabetes
Jørgensen *et al*[27], 1994, all strokes	233 diabetes 902 non-diabetes	ICH 1% *vs* 9% Infarct 60% *vs* 68%	Hypertension
Olsson *et al*[28], 1990, all strokes	121 diabetes 584 non-diabetes	ICH 6% *vs* 9% Infarct 59% *vs* 55%	Heart failure, ischaemic heart disease
Kiers *et al*[29], 1992, all strokes	27 diabetes 100 non-diabetes	ICH 19% *vs* 21% Infarct N/A	N/A
Weir *et al*[30], 1997, all strokes	61 diabetes 750 non-diabetes	ICH 7% *vs* 14% Infarct N/A	Hypertension, hyperglycaemia
Megherbi *et al*[31], 2003, all storkes	937 diabetes 3544 non-diabetes	ICH 8.5% *vs* 11.5% Infarct 78% *vs* 72%	Hypertension
Arboix *et al*[32], 2005, ischaemic strokes	393 diabetes 1447 non-diabetes	Infarct 76% *vs* 51%	Ischaemic heart disease, previous ischaemic stroke, dyslipidaemia
Hankey *et al*[33], 2013, all strokes	9795 diabetes	ICH 10% Infarct 82%	Hypertension, previous ischaemic stroke, ischaemic heart disease, nephropathy, high LDL cholesterol

[1]Prospective series reported in the literature. ICH: Intracerebral haemorrhage; LDL: Low-density lipoprotein; N/A: Not available.

stress leading to several pathological processes involved in diabetes-related microvascular complications[36]. Hyperglycaemia-induced overproduction of reactive oxygen species (ROS) inhibits the action of glyceraldehyde 3-phosphate dehydrogenase (GADPH), a key enzyme in glycolysis. When free radicals induce DNA strand break, ROS activates the DNA repair enzyme Poly(ADP-ribose) polymerase (PARP). Active PARP then modifies GAPDH and inhibits its activity. This results in the accumulation of glycolytic intermediates upstream of GAPDH which drive 5 pathogenic pathways contributing to endothelial dysfunction and diabetes complications: (1) polyol pathway flux; (2) increased formation of advanced glycation end products (AGEs); (3) increased expression of receptors for AGEs; (4) activation of protein kinase C isoforms; and (5) overactivity of hexosamine pathway[36].

Vasculopathy induced by chronic hyperglycaemia related endothelial damage results in acceleration of atherosclerosis inherent to diabetes. Therefore, higher prevalence and incidence of cardiovascular disease including stroke are common in the diabetic population.

Metabolic memory

The term "metabolic memory" is derived from the findings of DCCT/EDIC study and describes how the beneficial effects of immediate intensive treatment for hyperglycaemia is maintained for several years, regardless of future course of glycaemia[37,38]. More recent evidence indicates hyperglycaemia-induced ROS production triggers persistent epigenetic changes in nuclear factor-κB (NF-κB) within endothelial cells despite return to euglycaemic state. NF-κB mediates expression of inflammatory genes[39]. Epigenetic changes involve chromatin remodeling and changes in levels of gene expression[40]. This suggests even short-term hyperglycaemic spikes have a substantial impact on endothelial dysfunction independent of long-term glycaemic control. Switching off the metabolic memory effect of hyperglycemia-induced ROS is an important strategy in the prevention of cardiovascular complications related to diabetes.

Therefore, early management of hyperglycaemia in new onset diabetes should be advocated to halt the hyperglycaemia-induced pathological processes described earlier[36]. Unfortunately, maintaining good glycaemic control still does not prevent the progression of complications. So, new therapeutic strategies are being considered to prevent the overproduction of free radicals[39,41].

Insulin resistance

Insulin resistance plays a major role in the pathology of cardiovascular disease. In the context of excess adipose tissue, insulin is unable to suppress lipolysis activity, which results in free fatty acid (FFA) mobilization. The influx of FFA inhibits insulin stimulated peripheral glucose uptake in the liver, skeletal muscle, and other organs. In the vascular endothelial cells, FFA influx leads to mitochondrial overproduction of ROS, which activates the same pathogenic processes as hyperglycaemia. Increased FFA release also results in an adverse lipid profile characterized by raised triglycerides, reduced high-density lipoprotein cholesterol, and increased levels of small dense low-density lipoprotein (LDL) particles that accumulate in the arterial wall. In the context of insulin resistance, increased FFA and defective insulin signaling receptors on the macrophages contribute to macrophage apoptosis and poor clearance of LDL by phagocytosis. Consequently, necrotic breakdown of advanced lipid-rich plaques occurs, which lead to the progression of clinically relevant atherosclerotic lesions[39].

Preclinical studies have identified peroxisome-proliferator-activated receptor γ (PPARγ) in macrophage foam cells, endothelial cells, and smooth muscle cells in atherosclerotic lesions[42]. PPARγ is a nuclear receptor that regulates lipid metabolism and glucose homeostasis.

Thiazolidinediones, initially identified as drugs for T2DM by reducing systemic insulin resistance[43], are PPARγ ligands that have been shown to have protective effects against atherosclerosis progression in animal models and clinical studies[42,44]. Unfortunately, studies reviewing the use of thiazolidinediones in patients with T2DM have not consistently shown this effect[45,46]. The more recent Insulin Resistance Intervention after Stroke trial reviewed the use of the thiazolidinedione pioglitazone in patients without established T2DM but with markers of insulin resistance. Pioglitazone significantly reduced total cardiovascular events by 24% (HR = 0.76; 95%CI: 0.62-0.93, *P* =0.007), but was also associated with significant adverse drug effects contributing to nonadherence in the intervention arm[44,47].

GLYCAEMIC MANAGEMENT DURING THE ACUTE PHASE OF STROKE

Hyperglycaemia is frequently seen in acute stroke patients, irrespective of diabetes diagnosis, and it is associated with increased morbidity and mortality[30,48]. In many patients, the first diagnosis of diabetes is often made in the event of an acute stroke and especially in the elderly. Numerous observational studies have shown that acute hyperglycaemia in stroke is associated with larger infarct volumes, longer in-hospital stay, poor functional recovery, and increased 30-d mortality[33].

There is limited evidence to suggest active glucose reduction with intravenous insulin therapy improves stroke outcomes[49,50]. The largest efficacy trial to date, the United Kingdom Glucose Insulin in Stroke Trial, showed no difference in mortality or functional outcomes in patients with mild to moderate blood glucose elevations (median 7.8 mmol/L). Episodes of hypoglycaemia were also observed in 41% of subjects in the treatment arm. Therefore, the use of insulin infusion regimens with mild to moderate hyperglycaemia is not advisable. Current guidelines recommend maintaining blood glucose levels in range of 140-180 mg/dL (7.8-10.0 mmol/L), and it is common practice to use intravenous glucose/potassium/insulin (GKI) in the first 24 h after stroke[50-52].

The evidence of glycaemic management in the following days after a stroke is less clear as enteral feeding and oral intake can cause fluctuations in post-prandial glucose excursions. No randomised, prospective intervention studies have proven insulin administration for diurnal glycaemic variability translates to clinical benefits[53,54]. The Heart2D trial specifically reviewed the impact of prandial glucose spikes after an acute myocardial infarction and found that subcutaneous insulin regimens targeting prandial *vs* fasting glycaemic control in diabetes subjects did not result in any differences in risk for future cardiovascular events (HR = 0.98, 95%CI: 0.8-1.21)[54]. The use of subcutaneous or intravenous insulin or oral agents will need to be balanced with the clinical presentation and risk of hypoglycemia[52].

LONG-TERM GLYCAEMIC CONTROL

There is reasonable evidence to suggest a period of intensive glycaemic control results in sustained reduction of microvascular complications in those with T1DM and T2DM because of the effects on metabolic memory[38,41,55]. However, it is less clear how beneficial long-term glycaemic control is on cardiovascular outcomes including stroke[55-59].

The DCCT/EDIC study showed that intensive glycaemic control resulted in significant reduction in cardiovascular events in recently diagnosed T1DM subjects[38]. Study patients without any cardiovascular risk factors who were treated in the intensive arm had a 57% reduction in major cardiovascular disease outcomes during the 17 years of follow-up. This study suggested poor glycaemic control is associated with increased cardiovascular risk and intensive treatment reduces such risk in individuals with T1DM. Subsequent follow-up in the DCCT/EDIC cohort, now 27 years, demonstrates the continuing importance of early optimal glycaemic control with reduced overall mortality risk observed in the intensive group (*P* = 0.045), albeit with a small absolute risk reduction (approximately 1/1000 patient years)[58].

The increase and early risk of cardiovascular disease in T1DM has been well-documented in the literature. Even with early institution of intensive glycaemic control, its prevention and management require target-driven optimisation of individual cardiovascular risk factors (dyslipidaemia, hypertension, hypercoagulability, renal impairment). However, the specific risks toward cardiovascular disease in the T1DM population still needs to be elucidated, and active research in this patient group will be important in determining future clinical care as emphasized by the current AHA/ADA scientific statement on T1DM and cardiovascular disease[60].

The clinical relevance of glycaemia course in early diabetes diagnosis was further reviewed in T2DM. The delayed benefits of intensive glycaemic therapy observed in the DCCT/EDIC study was also seen in the 10-year follow-up of UKPDS. Newly diagnosed T2DM subjects in the intensive arm had a reduction in microvascular complications (15%, *P* = 0.01), myocardial infarction (15%, *P* = 0.01), and all-cause mortality (13%, *P* = 0.007). Stroke incidence, however, did not decline[55].

Since then, several studies addressed whether the degree of glycaemic control improved longer-term cardiovascular outcomes. The Veterans Affairs Diabetes Trial (VADT) showed intensive glycaemic control (1.5% HbA_{1c} reduction) was not associated with any significant difference in cardiovascular outcomes or in the rate of all-cause mortality (HR = 1.07; 95%CI: 0.81-1.42, *P* = 0.62) in poorly-controlled (baseline mean HbA1c 9.4%) veterans with established T2DM (mean, 11.5 years)[61].

To further evaluate the effects of lowering glucose to near-normal levels on cardiovascular outcomes, Action to Control Cardiovascular Risk in Diabetes (ACCORD)

Table 3 Blood glucose targets for non-pregnant adults with diabetes[1]

More stringent target (< 6.5%)
Short diabetes duration
Long life expectancy
T2DM treated with lifestyle or metformin only
No significant CVD/vascular complications
Less stringent target (< 8.0%)
Severe hypoglycaemia history
Limited life expectancy
Advanced microvascular or macrovascular complications
Extensive comorbidities
Long-term diabetes in whom general HbA1c targets are difficult to attain
Targets may be individualized based on:
Age/life expectancy
Comorbid conditions
Diabetes duration
Hypoglycaemia status
Individual patient considerations

[1]More or less stringent targets may be appropriate for individual patients if achieved without significant hypoglycaemia. CVD: Cardiovascular disease; T2DM: Type 2 diabetes mellitus; HbA1c: Glycated hemoglobin.

and Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trials were performed on subjects with long-standing T2DM (median 10 years duration) and already established cardiovascular risk[56,57]. Both ACCORD and ADVANCE did not demonstrate that intensive glycaemic control [HbA_{1c} < 42 mmol/mol (6.0%) and HbA_{1c} ⩽ 48 mmol/mol (6.5%) respectively] in the first few years significantly reduced cardiovascular events including strokes. Intensive therapy was associated with no risk reduction in non-fatal strokes in ADVANCE (HR = 0.97; 95%CI: 0.81-1.16) and a nonsignificant increase risk in ACCORD (HR = 1.06; 95%CI: 0.75-1.50, *P* = 0.74). The ACCORD trial also identified a clear difference in mortality within the first two years and was terminated early after demonstrating an increase in total (22%) and cardiovascular (35%) mortality rates in the intensive-therapy group[56].

Finally, a meta-analysis performed on 27,049 participants involved in UKPDS, ACCORD, ADVANCE, and VADT suggested a small reduction in major cardiovascular events (HR = 0.91, 95%CI: 0.84-0.99) but no difference in cardiovascular (HR 1.10, 95%CI: 0.84-1.42) or all-cause (HR = 1.04, 95%CI: 0.90-1.20) mortality[62]. These studies tell a cautionary tale and underscore how active intensification of glycaemic control can cause harm with early, increase mortality, particularly in T2DM patients with pre-existing cardiovascular disease.

Rather than treating a single factor, intensive intervention should include multiple risk factors that can influence cardiovascular outcomes and mortality. In support of this, the Steno-2 Study showed long-term (mean, 7.8 years), focused intervention for multiple risk factors (hyperglycaemia, hypertension, dyslipidaemia, BMI > 25, smoking) led to reduction of cardiovascular events among patients with established T2DM and microalbuminuria[63].

Table 4 Relative risk for ischaemic stroke incidence dependent on history of hypertension and diabetes at baseline[64]

Variables	Relative risk (95%CI)
Hypertension only (sBP 140-159 mmHg)	1.29 (1.13-1.46)
Hypertension only (sBP ⩾ 160/95 mmHg)	1.93 (1.48-4.16)
Diabetes only	2.48 (1.48-4.16)
Diabetes and hypertension (sBP 140-159 mmHg)	4.26 (2.90-6.25)
Diabetes and hypertension (sBP ⩾ 160 mmHg)	4.90 (3.87-6.21)

sBP: Systolic blood pressure.

There are several conclusions that can be inferred from these large, well-documented studies. Establishing good glycaemic control is certainly important in reducing diabetes complications, but there is no justification for targeting glucose levels to near-normal physiological parameters. Such an approach would not benefit patients with long-standing diabetes and established cardiovascular disease. HbA_{1c} reduction does not appear to be equally relevant in T2DM compared to T1DM in reducing stroke outcomes. The most appropriate target for HbA_{1c} should remain 53 mmol/mol (7%) with some caveat towards individualised targets as based on ADA guidelines summarised in Table 3[59]. More stringent targets may be appropriate, but requires an assessment to balance the expected benefits with the increased rates of adverse outcomes. Ultimately, the perception of diabetes management extends from hyperglycaemia and insulin resistance to considering other aspects of metabolic disorder which contribute to cardiovascular disease.

MANAGEMENT OF COMORBID CONDITIONS IN PATIENTS WITH DIABETES AND STROKE

Hypertension

Hypertension is a potent, treatable risk factor for stroke and more so in those with diabetes. Table 4 shows relative risk of stroke in patients with diabetes, hypertension or both[64]. In the DCCT/EDIC trial, higher HbA_{1c} was associated with a 25% increased risk of hypertension at EDIC follow-up (HR = 1.25; 95%CI: 1.14-1.37). However, intensive glycaemic therapy only reduced long-term risk of hypertension by 24% (HR = 0.76; 95%CI: 0.64-0.92). This suggests that standard cardiovascular risk factors gain more importance as glycaemic control improves[65].

Multiple studies have shown blood pressure (BP) control is important in reducing stroke risk in subjects with diabetes. In the UKPDS, T2DM patients in the tight control arm had a significantly lower BP (144/82 mmHg) compared with those in the standard control arm (154/87 mmHg) and this was associated with a

44% reduction in stroke[55].

Most guidelines, including AHA/ASA, recommend a BP target of < 140/90 mmHg in patients[66-68]. Lower targeted BP values have been evaluated with promising cardiovascular benefits but limited by adverse side effects, at least in diabetic patients at high risk of a cardiovascular event. The ACCORD BP trial reported intensive systolic BP (sBP) control to 120 mmHg, compared with a goal of 140 mmHg, among T2DM patients was associated with a significant reduction in total stroke (HR = 0.59; 95%CI: 0.39-0.89, P = 0.01) and nonfatal stroke (HR = 0.63; 95%CI: 0.41-0.96, P = 0.03)[69]. However, the intensive arm also had a significant number of adverse events. A meta-analysis in subjects with T2DM analyzed less modest BP targets than ACCORD and showed targeting a systolic BP ⩽ 135 mmHg resulted in a 17% risk reduction for stroke. Further meta-regression analysis showed continued risk reduction for stroke with a sBP of < 120 mmHg but even at levels < 130 mmHg there was a 40% increase in serious adverse events without any other cardiovascular benefits besides stroke[70].

There is enough evidence to suggest an antagonist of the renin-angiotensin system has cardiovascular benefits[71,72]. The Heart Outcomes Prevention Evaluation study reviewed the use of an ACE-inhibitor in high risk patients for cardiovascular event[71]. In the subgroup of patients with diabetes, there was a 25% reduction in primary outcome of MI, stroke, and cardiovascular mortality (95%CI: 12%-36%; P = 0.0004) in the ACE-inhibitor treated arm.

Overall, these studies suggest hypertension management (BP < 140/90 mmHg) improves stroke risk in subjects with diabetes independent of glycaemic control. Young people with diabetes and those with microalbuminuria should aim for BP control ⩽ 130/80 mmHg. A more aggressive approach targeting systolic BP < 120 mmHg in patients already at high risk for a cardiovascular event can be limited by adverse side effects and does not translate to further reduction in cardiovascular outcomes besides stroke.

Pharmacotherapy should include an antagonist of the renin-angiotensin system (unless contraindicated), either an ACE-inhibitor or an angiotensin-receptor blocker but not both[67,73]. Other common antihypertensive agents include calcium channel antagonists, beta blockers and diuretics. AHA/ASA guidelines recommend the choice of antihypertensive be individualised to the patient with specific consideration based on clinical indication[68].

Obesity

Obesity is a growing epidemic in developed and developing countries. The proportion of adults with a body mass index (BMI) ⩾ 25 kg/m^2 has increased from 28.8% (95%CI: 28.4-29.3) in 1980 to 36.9% (36.3-37.4) in 2013 in men and from 29.8% (29.3-30.2) to 38.0 (37.5-38.5) in women[74]. Obesity increases the risk of T2DM, ischaemic heart disease, stroke, and mortality[75-77]. It is also associated with the metabolic syndrome, which is a constellation of cardiovascular factors including dyslipidaemia, hypertension, hyperinsulinaemia, and insulin resistance[78].

Weight reduction of ⩾ 5% of initial body weight improves control of diabetes and hypertension, reduces risk of diabetes and hypertension incidence, and reduces other metabolic risk factors[79,80]. The difficulties faced in any weight loss intervention is ensuring this can translate to long term health benefits. The Look AHEAD research group evaluated the role of Intensive lifestyle intervention which included a healthy diet with a calorie goal of 1200 to 1800 kcal per day (with < 30 % of calories from fat and > 15% from protein) and at least 175 min of moderate-intensity physical activity per week in contributing to weight loss[81]. They observed that intensive lifestyle intervention resulted in greater sustained weight loss than in the control group (8.6% *vs* 0.7% at 1 year; 6.0% *vs* 3.5% at study end). However, this weight loss did not reduce the rate of cardiovascular morbidity and mortality in overweight or obese adults with T2DM at 10-year follow-up (HR = 0.95; 95%CI: 0.82-1.09; P = 0.51).

The degree of long-term weight reduction may be important to overall cardiovascular benefit. The Swedish Obese Subjects study had shown cardiovascular risk factor improvement over 10 years required sustained, large (*i.e.,* 10-40 kg) weight loss that could not be achievable with intensive lifestyle intervention alone[82,83]. Metabolic surgery has been associated with reduced number of cardiovascular deaths (HR = 0.47; 95%CI: 0.29-0.76; P = 0.002) and reduced total first incidence (fatal or nonfatal) of myocardial infarction or stroke (HR = 0.67; 95%CI: 0.54-0.83; P < 0.001)[84].

Most efforts to achieve sustainable weight reduction with lifestyle intervention and medical therapy have been unsuccessful. Lifestyle intervention still conveys other potential benefits by improving physical functioning and quality of life; therefore, it is integral for good health outcomes[3]. Pharmacotherapy for glucose management should consider weight loss or weight neutral medications in preference to those promoting weight gain. Concomitant medications should be rationalized to minimize weight gain[85]. Metabolic surgery for obese individuals with T2DM has shown cardiovascular benefits and is an important clinical consideration in obese (BMI > 40) T2DM individuals[86]. Identifying new pathways leading to safe and effective weight reduction continues to be sought. In recent years, there has been focus in gene variants predisposing individuals to type 2 diabetes and obesity[87]. Investigators from the Look AHEAD trial reported how genetic variants can help predict cardiovascular morbidity and mortality[88]. Such information on genetic studies continues to be garnered and can potentially allow for new targets for pharmaceutical intervention in the future[87,88].

Dyslipidaemia

The Heart Protection Study and Collaborative Atorvastatin Diabetes Study have demonstrated how statins

Table 5 Trials of statin therapy with individual participant data and relative reduction of cardiovascular event rate including stroke

Study	Randomized participants, age	Type of Prevention	Diabetes participants (%)	Intervention (mg/d)	Follow-up (yr)	Relative reduction of CVE rate
4S[92,93]	4444, 35-70 years old	Secondary	202 (4.50%)	S20-40	5.4	37%
CARE[94,95]	4159, 21-75 years old	Secondary	586 (14.10%)	P40	5.0	25%
LIPID[91,96]	9014, 31-75 years old	Secondary	1077 (11.9%)	P40	6.1	21%
ALLHAT-LLT[97]	10355, ⩾ 55 years old	Primary	3638 (35%)	P40	4.8	11%
HSPC[89]	20536, 40-80 years old	Primary, secondary	5963 (29%)	S40	4.8	22% total 33% primary
ASCOT-LLA[98,99]	19342, 40-79 years old	Primary	2532 (13%)	A10	3.3	23%
CARDS[90]	2838, 40-75 years old	Primary	2838 (100%)	A10	3.9	37%

CVE: Cardiovascular event; S: Simvastatin; P: Pravastatin; A: Atorvastatin.

improve cardiovascular risk in patients with diabetes by lowering LDL cholesterol[89,90]. Stroke incidence was significantly higher among those with diabetes and impaired fasting glucose, and treatment of dyslipidaemia was more effective for secondary prevention in these groups compared to subjects with normal fasting glucose[91]. Statin therapy should now be considered routinely for all diabetes patients beyond 40 years of age and earlier in high risk groups, irrespective of their initial cholesterol concentrations. Table 5 summarises major clinical trials showing the benefits of statin therapy in diabetes participants[92-99].

Ezetimibe with statin therapy can provide additional cardiovascular benefits as it reduces LDL cholesterol levels by a further 24%. A recent study showed cardiovascular benefits with this dual therapy particularly in patients with a recent acute coronary syndrome[100]. The addition of fibrate has not been shown to significantly improve cardiovascular outcomes but it can be considered in a subgroup of T2DM subjects with mixed dyslipidaemia[101].

Atrial fibrillation

Atrial fibrillation (AF) is associated with a 4- to 5-fold increase risk for ischaemic stroke[102]. In patients with AF, clinical predictive risk scores have been useful in stratifying patients for anticoagulation therapy. The primary example is the CHA_2DS_2-VASc score [congestive heart failure, hypertension, age ⩾ 75 years (doubled), diabetes, stroke/transient ischaemic attack/thromboembolism (doubled), vascular disease, age 65-74 years, sex (female)] which has been recommended in clinical practice guidelines[103,104].

Diabetes has been associated with an increased risk of developing persistent AF[105]. A meta-analysis reviewed this association and reported that approximately 25% of diabetes patients will have AF[106]. The relevance of diabetes with AF on stroke risk is not clearly determined, although a diagnosis of diabetes is included in the CHA_2DS_2-VASc score[107]. To ascertain whether aspects of diabetes influence risk, a study reviewed the role of glycaemic control and duration of diabetes on stroke risk in subjects from the ATRIA (Anticoagulation and Risk Factors in Atrial Fibrillation) cohort during a period off anticoagulation therapy[108]. The study reported an increased rate of ischaemic stroke with longer duration of diabetes (adjusted HR = 1.74, 95%CI: 1.10-2.76) but not with increased HbA1c. Further studies reviewing aspects of diabetes may prove useful in stratifying stroke risk in AF patients and refining current stroke risk models.

Clinical trials and practice-based experience with newer oral anticoagulants (NOAC) such as rivaroxaban, edoxaban, apixaban and dabigatran in recent years showed beneficial effects in prevention of stroke in patients with AF. Ease of administration without regular monitoring, better patient adherence, and probable improved efficacy and safety make NOAC more favourable to physicians in treating AF and venous thromboembolism in the present day clinical practice[109]. Although there is no data to show higher efficacy of NOAC in comparison with warfarin for stroke prevention in patients having AF with or without diabetes, better patient adherence and therefore, possibly improved clinical outcomes are shown in recent studies[110,111].

Heart failure

Incident heart failure is also associated with increased risk of ischaemic stroke, even without atrial fibrillation[112,113]. Heart failure is common in subjects with diabetes that is associated with higher risk for stroke. A recent study demonstrated heart failure patients with diabetes and no AF was associated with a 27% increased relative risk of ischaemic stroke[114]. While this study tried to stratify the degree of risk by duration of diabetes, no clear relationship could be elucidated; this may be attributed to the limited sample sizes in the subgroups and the short follow-up time.

The CHA_2DS_2-VASc score, as discussed previously, is applied for stroke risk stratification in atrial fibrillation. A study reviewed CHA_2DS_2-VASc score in patients with heart failure without atrial fibrillation and found that the absolute risk of thromboembolic complications was higher in this group compared to patients with concomitant AF[115]. Currently, patients with heart failure and no AF are not routinely recommended to take antiplatelet or anticoagulation therapy. If further studies support the finding of increased stroke and thromboembolic disease in heart failure patients with diabetes, consideration of anticoagulation in a subgroup of these patients may be

clinically relevant.

Antiplatelet therapy

Antiplatelet therapy significantly reduces recurrent cardiovascular events outcomes among patients with diabetes. The CAPRIE trial demonstrated clopidogrel is superior to aspirin in reducing cardiovascular events and causing few bleeding complications in diabetic patients with established atherosclerotic disease[116]. Unfortunately, these clinical benefits do not extend to primary prevention. Short-term dual therapy with Aspirin and Clopidogrel improves stroke outcomes in patients presenting with an acute TIA or minor stroke[117]. The use of long-term dual therapy is still unclear; while there may be a relative risk reduction of stroke this is unbalanced by the increased haemorrhagic risk[118].

CAROTID ENDARTERECTOMY

Some patients with symptomatic carotid stenosis would benefit from surgical intervention. Carotid endarterectomy appears to reduce the risk of stroke in diabetic patients with severe stenosis (*i.e.,* $\geqslant$ 70% stenosis) on long-term follow up[119]. Mild to moderate stenosis (*i.e.,* < 70%) was not associated with such clinical benefits. However, a recent study demonstrated that diabetes with chronic complications increased the risk for myocardial infarction, stroke, perioperative infections, longer hospital stay and mortality compared to nondiabetics treated with carotid endarterectomy although diabetics without complications did not show this risk[120].

DIABETES AND STROKE: RECENT DEVELOPMENTS

With the emergence of newer oral and injectable anti-diabetic agents in the management of T2DM, the use of older agents with hypoglycaemia risk such as insulin and sulphonylureas as well as glinides is less favoured by physicians recently. While metformin and pioglitazone have demonstrated cerebrovascular benefit in the insulin resistant population, the GLP-1 analogues have proved their efficacy in cardiovascular outcomes along with weight and blood pressure reduction. The EMPA-REG Trial showed significant cardiovascular benefit with weight and blood pressure reduction though there was a marginal signal of higher stroke rates[121]. Improvement of renal outcomes was another promising benefit of empagliflozin use demonstrated recently that may translate into better cardiovascular outcomes in T2DM patients with diabetic nephropathy[122].

The newer cardiovascular outcome trials LEADER and SUSTAIN-6 using GLP-1 analogues have shown reduction in stroke and cardiovascular event risk as well as lower nephropathy and hypoglycaemia rates in patients with longstanding diabetes and very high cardiovascular risk adding to the armamentarium of agents with low risk of hypoglycemia or weight gain[123,124]. Technological advances in insulin delivery and glucose monitoring have improved the prospects of glycaemic management of T1DM and may reduce the future risk of stroke.

The United Kingdom National Clinical Guidelines for Stroke have been recently updated and provide an elaborate care plan for patients with stroke[125]. Individualised care plan for stroke patients depending on the clinical scenario should be tailored with considerations of disease co-morbidities including diabetes. An up to date scientific evidence should always lead the clinicians to optimise such care plan.

CURRENT RECOMMENDATIONS FOR MANAGEMENT OF CARDIOVASCULAR RISK FACTORS IN PATIENTS WITH DIABETES

The Joint European-American diabetes guidelines has given a nice illustration of the target HbA1c in different scenarios[126]. While aiming for a tighter control in those who are young, fit, and very motivated with recent onset diabetes and on agents with no risk of hypoglycemia, a less stringent target should be adopted for those who are frail, elderly and with long duration of diabetes on agents causing hypoglycemia as well as those with cognitive decline. Randomised controlled trials ACCORD and ADVANCE have shown that it is safe to aim for a HbA1c of 53 mmol/mol (7.0%) in those with long duration of diabetes and established cardiovascular disease rather than tighter control[56,57]. An evidence-based recommendation for management of patients with cardiovascular risk factors is summarised in Table 6[127-129].

CONCLUSION

The incidence of stroke and its sequelae are on the rise. Patients with diabetes are particularly at a significantly higher risk of stroke and have a higher mortality. Initiating good glycaemic control at first diagnosis of diabetes, irrespective of type, is essential for sustained cardiovascular benefits (*i.e.*, metabolic memory) and for the reduction of hyperglycaemia-induced pathogenic processes implicated in atherosclerotic vascular disease. However, long term tight glycaemic control has not been shown to improve cardiovascular outcomes and therefore, subsequent management should focus on modifiable cardiovascular risk factors. We have summarised a few recommendations with relevant supporting literature to help clinicians to approach patients with diabetes and stroke as outlined in Table 6. As the population is ageing, the "time-bomb" of diabetes in older people is becoming more and more obvious. The economic, physical, medical, nursing, and psycho-social implications of diabetes and stroke will be immense

Table 6 Recommendations for cardiovascular risk factor management in patients with diabetes

Condition	Supporting literature
Hyperglycaemia	
Targeting HbA1c < 6.5% to reduce cardiovascular events is not beneficial and is harmful when compared with a target of 7.0%	ACCORD[56], ADVANCE[57]
Hypertension	
BP < 140/90 mmHg improves risk of cardiovascular and cerebrovascular outcomes (33)	UKPDS[55]
Targeting sBP < 120 does not improve cardiovascular outcomes and is associated with increased risk of adverse side effects	ACCORD-BP[69]
Antagonist of renin-angiotensin system is associated with cardiovascular benefits	HOPE[71]
Dyslipidaemia	
All patients age > 40 yr, with or without history of atherosclerotic vascular disease, should receive statin therapy	HPSC[89], CARDS[90]
Use of ezetimibe with statin therapy can improve cardiovascular outcome in patients with a recent acute coronary syndrome and LDL > 50 mg/dL (1.3 mmol/L)	IMPROVE-IT[100]
Use of fibrates may be effective in selected patients with HDL < 34 mg/dL (0.9 mmol/L) and triglycerides > 204 mg/dL (2.3 mmol/L)	FIELD[101]
Obesity	
Intensive lifestyle intervention with diet, physical activity, and medical therapy improves quality of life and physical function	Look AHEAD[81]
Metabolic surgery has been shown to improve long-term cardiovascular outcomes	SOS[82]
Antiplatelet therapy	
Aspirin use in acute coronary syndrome treatment and in secondary prevention has been established	ISIS-2[127]
Clopidogrel use in secondary prevention reduces more cardiovascular outcomes and causes fewer bleeding complications compared to aspirin in diabetic patients	CAPRIE[116]
In patients with acute TIA or minor stroke, combination of clopidogrel and aspirin is superior to aspirin alone for reducing risk of stroke in the first 90 d without increasing risk of haemorrhage	CHANCE[117]
Use of aspirin in primary prevention has not been shown to improve cardiovascular outcomes	JPAD[128]
Low-dose aspirin use for primary prevention of cardiovascular disease in adults who have a 10% or greater 10-yr cardiovascular risk, are not at increased risk of bleeding, and are willing to take daily aspirin for at least 10 yr	USPSTF[129]

ACCORD: Action to Control Cardiovascular Risk in Diabetes; UKPDS: United Kingdom Prospective Diabetes Study; ACCORD-BP: Action to Control Cardiovascular Risk in Diabetes-Blood Pressure; HOPE: Heart Outcomes Prevention Evaluation; CARDS: Collaborative AtoRvastatin Diabetes Study; FIELD: Fenofibrate Intervention and Event Lowering in Diabetes; Look AHEAD: Look Action for HEAlth in Diabetes; CAPRIE: Clopidogrel *vs* Aspirin in Patients at Risk of Ischemic Events; CHANCE: Clopidogrel in High-risk patients with Acute Nondisabling Cerebrovascular Events; JPAD: Japanese Primary Prevention of Atherosclerosis with Aspirin for Diabetes; USPSTF: United States Preventive Services Task Force;; sBP: Systolic blood pressure; LDL: Low-density lipoprotein; HDL: high density lipoprotein.

in the future. Health authorities and policy makers throughout the world will need to pay special attention to the duo of diabetes and stroke to alleviate or prevent the resultant complications.

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REVIEW

Diabetes-induced mechanophysiological changes in the small intestine and colon

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Abstract

The disorders of gastrointestinal (GI) tract including intestine and colon are common in the patients with diabetes mellitus (DM). DM induced intestinal and colonic structural and biomechanical remodeling in animals and humans. The remodeling is closely related to motor-sensory abnormalities of the intestine and colon which are associated with the symptoms frequently encountered in patients with DM such as diarrhea and constipation. In this review, firstly we review DM-induced histomorphological and biomechanical remodeling of intestine and colon. Secondly we review motor-sensory dysfunction and how they relate to intestinal and colonic abnormalities. Finally the clinical consequences of DM-induced changes in the intestine and colon including diarrhea, constipation, gut microbiota change and colon cancer are discussed. The final goal is to increase the understanding of DM-induced changes in the gut and the subsequent clinical consequences in order to provide the clinicians with a better understanding of the GI disorders in diabetic patients and facilitates treatments tailored to these patients.

Key words: Diabetes; Intestine; Colon; Biomechanics; Motor-sensory; Gut microbiota; Symptoms

Core tip: The disorders of intestine and colon are common in patients with diabetes mellitus (DM). DM induced intestinal and colonic structural and biomechanical remodeling are closely related to motor-sensory abnormalities of gut in DM. These changes due to DM are associated with diarrhea, constipation, gut microbiota modification and colon cancer. Understanding the DM-induced changes in the gut and the clinical consequences provides clinicians with a better understanding of the gastrointestinal disorders in diabetic patients and facilitates the improvement of treatments for these patients.

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INTRODUCTION

Diabetes mellitus (DM) is a popular metabolic disease which affects many populations worldwide[1]. Complications in different organ systems including the gastrointestinal (GI) tract will occur if the DM is treated inappropriately. NCD Risk Factor Collaboration has demonstrated that the number of adults with DM in the world increased from 108 million in 1980 to 422 million in 2014[1]. Furthermore, huge healthcare expenditures are needed in order to prevent and treat DM and its complications[2].

DM patients often suffer from GI disorders which are recently recognized as one of the most common complications in DM[3]. The whole GI tract can be affected in the DM and common complaints include diarrhea, constipation and fecal incontinence[4]. The symptoms are usually non-specific, but occasionally they may be severe enough to decrease the quality of life. The pathophysiological mechanisms of the symptoms are very complex; they may involve multiple factors and are inadequately explored. However, it is well known that the motor-sensory dysfunctions often seen in the DM patients are closely associated with diabetic autonomic neuropathy (DAN)[5-7]. Furthermore, it has been recently recognized that DAN also includes the disorders of the enteric nervous system (ENS)[7]. It is well known that DM induces histomorphological and biomechanical remodeling of small intestine and colon in type-1 DM patients[5] and in DM animals[8-10]. Such remodeling is closely related to motor-sensory dysfunctions in DM patients[9]. Understanding the mechanisms of DM-induced changes of the intestine and colon is of key importance for the optimization of treatment and for finding new therapeutic approaches.

In this review, we discuss: (1) DM-induced intestinal and colonic histomorphological changes and biomechanical remodeling; (2) intestinal and colonic sensory-motor dysfunction in relation to DM and its relation to the remodeling of intestine and colon; and (3) the clinical consequences of DM-induced changes in intestine and colon including diarrhea, constipation, GM change and colon cancer. It is well known that esophageal and gastric motility disorders are also very common in DM patients; however, these have been reviewed in detail recently (see references[11,12]). Furthermore, as we focus on the topic of DM-induced mechanophysiological changes in the small intestine and colon, the topic of esophageal and gastric disorders in the DM are not included in this review.

NORMAL ANATOMY, STRUCTURE AND BIOMECHANICAL PROPERTIES IN THE INTESTINE AND COLON

Anatomy and structure of normal intestine and colon

Understanding the anatomy and structure of normal intestine and colon is essential in order to gain an insight into the biomechanical properties and the DM-induced remodeling. Intestine and colon are tubular organs. From proximal to distal, the intestine consists of duodenum, jejunum and ileum. The ligament of Treitz marks the anatomical demarcation between duodenum and jejunum, whereas there is no distinct demarcation between jejunum and ileum. A mesentery anchors the jejunum (proximal 40%) and ileum (distal 60%) to the posterior wall of abdomen and allows the intestine to be freely moveable within the peritoneal cavity. The distal end of the intestine is in continuity with colon and the transition is marked by the ileocecal valve which prevents the retrograde flow of colonic contents into the small intestine[13]. The colon is composed of five parts namely cecum, ascending colon, transverse colon, descending colon and sigmoid colon. The external appearance of colon is distinctly different from that of the intestine. The longitudinal layer of muscle fibers forms three discrete bands named tenia, and the formation of sacs filled with adipose tissue on the inner surface gives the colon a segmented appearance characterized by small pouches named haustra.

The histologic characteristics of the intestine and colon shares many similarities. The wall is composed of four layers: Mucosa, submucosa, muscularis, and serosa with the mucosa being the innermost layer. The mucosa consists of sublayers of glandular epithelium, lamina propria, and muscularis mucosae. The glandular epithelium forms cylindrical structures called crypts. The lamina propria serves to support the epithelium and consists of reticular connective tissue with elastin, reticulin, and collagen fibers and cellular components such as lymphocytes, plasma cells, and eosinophilic granulocytes, as well as lymphatics and capillaries. The muscularis mucosae is a thin layer of smooth muscle intertwining the mucosa and submucosa. The submucosa is a fibrous connective tissue layer that contains fibroblasts, mast cells, blood and lymphatic vessels, and extraordinarily an autonomous nerve plexus called the Meissner's plexus which consists of non-myelinated, postganglionic sympathetic fibers and parasympathetic ganglion cells[14,15]. The muscularis propria beneath the submucosal layer consists of smooth muscle fibers and is responsible for the contractility of the intestines. The muscle fibers are arranged in a helicoidal pattern in two layers, an inner circular layer and an outer longitudinal layer. Between these two muscle layers there is a second autonomous nerve plexus named the myenteric plexus or Auerbach's plexus[15,16]. Parasympathetic and postganglionic sympathetic fibers in the plexus

terminate in parasympathetic ganglion cells; from here, the postganglionic parasympathetic fibers terminate in smooth muscle and influence the intestinal contractility by the release of neurotransmitters. The serosa is the outermost layer of the intestines and consists of connective tissue. Interstitial cells of Cajal (ICC) are present in both the small intestine and the colon and influence the contractility of the smooth muscle fibers. These cells act as pacemaker cells and are located in the myenteric plexus, the muscularis propria and the submucosa[17]. The ICCs express the receptor for tyrosine kinase (c-kit). Thus, immunohistochemical stains that utilize antibodies against c-kit allow the ICCs to be labeled[18].

Biomechanical properties of normal intestine and colon

One important function of both small intestine and colon is the transportation of food by peristaltic contraction. Furthermore, the mixing function by segmental contraction is also important for small intestine in order to establish close contact between the food and mucosa and fully absorb the nutritional contents. Both types of contraction are involved in the force (stress) changes and deformation (strain) in the wall of intestine and colon. Therefore, understanding the normal biomechanical properties is essential for the understanding of the physiological functions of small intestine and colon. Descriptions of biomechanical properties include elasticity such as tension-strain or stress-strain relations, and viscoelasticity such as creep and stress relaxation. Generally the biomechanical properties of small intestine and colon display an exponential behavior and are anisotropic with large axial and location variations[19-40]. The biomechanical characteristics of the normal small intestine and colon can be summarized in Table 1.

The variation of biomechanical properties along and across the wall of intestine and colon have important physiological significance. The residual strain makes the stress distribution through the wall more uniform in the pressurized state[27]. The compression residual stress reduces the stress concentration at the inner wall, thereby offering a better protection of intestine and colon against injury due to contractile activity and against the flow of luminal contents. Furthermore, the different stiffness has been demonstrated in different segments of intestine and colon. For example, the duodenal segment is stiffest whereas the ileum is softest in the small intestine. These may relate to the specialized functions in the proximal and distal locations. Duodenum acts as a capacitative resistor during gastric emptying whereas the transit of distal ileum is slow and acts as a reservoir[32]. The flow patterns of intestine may also relate to biomechanical properties. The stiff duodenal wall will be in favor of a lesser degree of bolus passing whereas the soft ileal wall will be in favor of pooling of luminal content and decreased flow.

DIABETES-INDUCED HISTOMORPHOLOGICAL AND BIOMECHANICAL CHANGES IN THE SMALL INTESTINE AND COLON

Histomorphological remodeling

DM-induced histomorphological changes involve different tissue components of the intestinal and colonic wall including epithelia, smooth muscle cell (Figure 1A, Figure 2), neurons, ICC and extracellular matrix (Table 2). Many animal and human studies have demonstrated that DM generally induces changes in the proliferation of different layers[5,8,10,41-51]. Increased expression of advanced glycation end of product (AGE) and AGE receptor (RAGE) has been demonstrated in the DM intestinal and colonic wall[49,51,52]. Furthermore, the number and density of neurons and ICC are changed, and the expressions of some neuropeptides alter as well (Table 2).

Biomechanical remodeling

In comparison with DM-induced histomorphological remodeling, there are not so many data in relation to the biomechanical remodeling in the small intestine and colon. Data on tension-strain relations has demonstrated that the stiffness of wall in the rat jejunum and ilem increases in DM rats[53]. Lately, the research group of Zhao *et al*[8,25] and Sha *et al*[48] did a series of studies investigating the histomorphological and biomechanical remodeling of small intestine in STZ-induced DM rats. They found in diabetic rats that (1) the opening angle and residual strain became smaller in the duodenum and larger in the jejunum and ileum; (2) the stiffness of the intestinal wall increased as function of time of DM development (Figure 1B and C); and (3) the stress of intestinal wall relaxed less(Figure 1D). More recently, remodeling of the jejunal wall in type 2 DM rats (GK rat) has been reported[47]. It was shown that the opening angle and residual strain were reduced and the wall stiffness increased in the circumferential direction. Furthermore, we demonstrated that increasing blood glucose level and the increased AGE/RAGE expression were associated with the remodeling. However, data on biomechanical changes in the diabetic colon is sparse. We have also investigated DM-induced biomechanical and morphometric remodeling in rat colon[10]. It was found in diabetic colon that the opening angle and residual strain became bigger and the stiffness of the colon wall increased with the duration of DM both in the circumferential and longitudinal directions (Figure 2). More recently, the remodeling of the distal colon in DM was studied by Siegman *et al*[51] in rats. A major finding from the study was the marked decrease in resting compliance and increase in stiffness of the smooth muscle cells of the distal colon in DM rats. Such changes are associated with increased production of type 1

Table 1 Biomechanical properties of normal small intestine and colon

	Biomechanical properties
Intestine	Tension-strain or stress-strain curves show an exponential behavior[19-23] The stiffness differs between the duodenal, jejunal and ileal segments[20,21,24] All segments are stiffest in longitudinal direction[20,21,24] The opening angle and residual strain shows a large axial variation[25]. The axial variation correlates to the morphometric variation[26] The serosal residual strains are tensile and the mucosal residual strains are compressive[24,25,27] The residual strains in longitudinal direction are smaller than those in circumferential direction[24], especially on the mucosal side The opening angle changes over time for all the small intestine segments. The viscoelastic constant of the rat small intestine is fairly homogenous along its length[28] The collagen in submucosa layer is important for the passive biomechanical properties[29,30] The villi are important for the biomechanical properties of the small intestine in circumferential direction[31]
Colon	The rat colon has a tensile strength of around 50 g/mm^2 and increases in strength from proximal to distal[33] Quasi-static P-V curves in colon are approximated to a power exponential function and revealed hysteresis, indicative of viscoelasticity[34] The opening angle vary along the rat colon with the highest values in the beginning of the proximal colon[35]. The residual strain is negative at the inner surface and positive at the outer surface[35] The stress-strain curves are exponential. All segments were stiffer in longitudinal direction than in the circumferential direction[35] In human sigmoid colon, the spatial distributions of the biomechanical parameters are non-homogeneous. The circumferential length, strain, pressure and wall stress increase as a function of bag volume[36] The wall stiffness of human sigmoid colon is reduced in response to butylscopolamine[36] The phasic and tonic responses to the meal in two colonic regions of human are quantitatively different but qualitatively similar[37] Smooth muscle cells in the gastrointestinal tract are constantly being deformed due to forces generated by the muscle cells themselves or by the surroundings[38,39] A mechanical creep behavior in the isolated rat colon smooth muscle cells could be described by a viscoelastic solid model[40]

Table 2 Diabetes mellitus-induced histomorphological changes of intestine and colon

	Intestine	Colon
Mucosa	Increased thickness[5,8,47,49]; Damaged tight junctions[260]; Proliferation of villi and crypt[41]; Decreased membrane fluidity[110]; Enhanced transport of glucose, amino acid, bile salts, phosphate, fatty acids, fatty alcohols, and cholesterol[110]; Decreased protein synthesis[261]; Increased expression of the monosaccharide transporters[262,263]; Increased expression of AGE and RAGE[47,49]	Increased thickness[10,49]; Increased thickness of the subepithelial collagen layer[276,277]; Abnormalities of endocrine cells[278]; Increased expression of RAGE[49]; Increased expression of AGE, RAGE, TGF-β1 and TGF-β1 receptor[52]
Submucosa	Increased thickness[5,8,47]	Increased thickness[10]; Increased expression of AGE, RAGE, TGF-β1 and TGF-β1 receptor[52]
Muscle	Increased thickness[8,47]; Increased expression of AGE and RAGE[49]	Increased thickness[10]; Hypertrophy of smooth muscle cells[51]; Increase type I collagen and expression of AGE[51]; Increased expression of AGE, RAGE, TGF-β1 and TGF-β1 receptor[52]
Wall as a whole	Increased thickness[8,47-50]; Increase expression of substance P[264] and neuronal nitric oxide synthase[265]; Dcreased expression of substance P[266], vasoactive intestinal polypeptide[262] and neuronal nitric oxide synthase[267]; Increased RAGE mRNA level[50]	Increased thickness[10,49]; Increase in substance *P* levels[264]
Nerve and ICC	Nuroaxonal dystrophy[48,268]; Decreased myenteric ganglia[269]; Decreased nitrergic neuronal cell number[270]; Decreased density of myenteric neurons[120]; Decreased number of myenteric neurons[271,272]; Increased expression of RAGE[49]; Decreased myosin-V-immunoreactive neurons[273]; Decreased ghrelin cell density[274]; Reduced number of ICC[99,275]	Impairment of nitrergic enteric neurons[111]; Decrease density and size of the myenteric neurons[15]; Decreased nitrergic neuronal cell number[280]; Decreased the numbers of nNOS, CHAT neurons and total neurons[279]; Increased expression of RAGE[49]; Apoptosis of neurons[244]; Decreased ghrelin cell density[274]; Reduced number of ICC[99,110,124,280] and impairment in the ultrastructures of ICC[99]

AGE: Advanced glycation end of product; RAGE: Advanced glycation end of product receptor; ICC: Interstitial cells of Cajal.

collagen and AGEs.

Mechanisms of histological and biomechanical remodeling

Hyperphagia: There is study which suggests that hyperphagia is related to DM-induced GI growth[54]. However, other researchers have found that when DM rats and normal rats are fed with same caloric diets, the intestinal mass and DNA synthesis in crypt still increases considerably in diabetic rats[55,56]. This indicates that DM-induced GI growth depends not only on increased nutrient consumption but also on other adaptation factors. It has been demonstrated that there is a close relation between glucagon-like peptide-2 (GLP-2) and DM-induced GI growth[57]. Increasing blood GLP-2 could precede the changes of intestinal mass[57]. Therefore, the increased nutrient in DM-induced GI growth may relate to its role in the stimulation of hormonal release

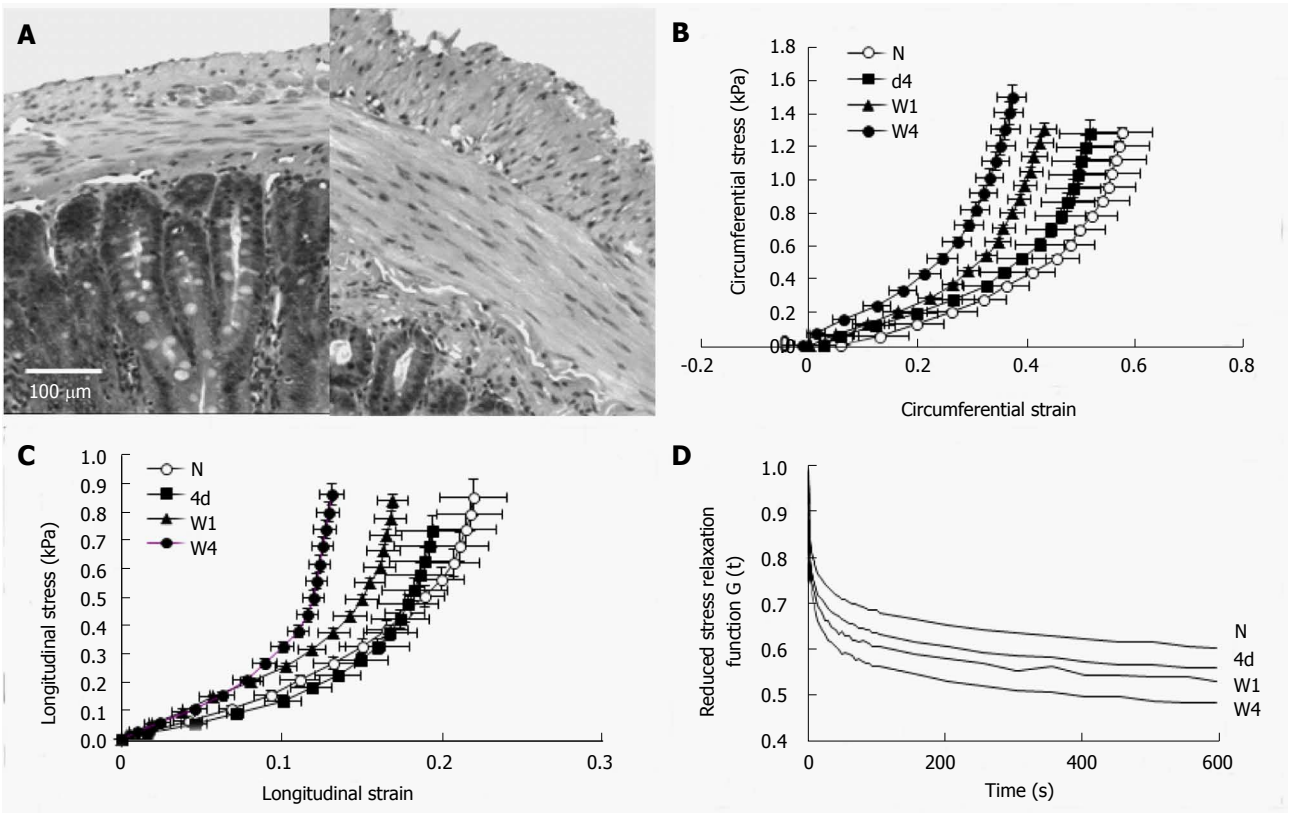

Figure 1 Duodenal remodeling in STZ-induced diabetic rats. A: The micro-photographs showed the normal (left) and 4 wk diabetic (right) duodenal histological sections. It clearly demonstrated that the muscle and submucosa layers in the diabetic duodenum became much thicker than in the normal duodenum. The bar is 100 μm; B: The circumferential stress-strain relations; C: The longitudinal stress-strain relations. The stress-strain curves in both directions (B and C) shifted to the left during experimental diabetes indicating the duodenal wall became stiffer during the development of diabetes; D: The mean reduced relaxation function curves in the time period of 600 s. The curves appear in the order of largest-to-smallest G (t) as W4, W1, 4d and N. The stress relaxation of duodenum decreased with the development of experimental diabetes. N: Normal control; 4d: 4 d of diabetes; W1: 1 wk of diabetes; W4: 4 wk of diabetes; W8: 8 wk of diabetes.

in the GI tract. The nutrient content in the small intestine is greatly increased due to hyperphagia and fast gastric emptying[56] in DM rats. It is well known that the luminal nutrients such as fat and carbohydrate could stimulate physiological L cells[58], therefore the increased luminal nutrients could stimulate GLP-2 secretion and its action on the intestinal epithelium. Furthermore, the balance of the epithelial homeostasis is regulated by cell proliferation and death. It has been shown that apoptosis is inhibited in DM rats which in turn results in the increase of mucosal mass in the small intestine[44].

Non-enzymatic glycation of protein: Hyperglycemia is the most important feature of DM. The increased glucose can induce AGEs formation through non-enzymatic glycation of protein amino groups and by oxidation reaction[59]. The overproduction and accumulation of AGEs in the tissues could alter the structure and function of proteins[60] in the intestinal wall such as collagen. Such changes cause cross-linking of collagen, basement membrane thickening and the loss of matrix elasticity[61-63]. AGEs and corresponding receptors (RAGE) have been demonstrated to be up-regulated in the GI tract both in the experimental type 1[49] and type 2[64] DM rats. Furthermore, there is an association between AGEs and RAGE with DM-induced intestinal and colonic remodeling[47,51]. Two major mechanisms are mainly involved in the link between AGEs and DM-induced GI morphological and biomechanical remodeling. One is a receptor-independent pathway where the AGEs induce changes in the extracellular matrix architecture through the formation of protein cross-links. The other is a receptor-dependent pathway where the AGEs modify cellular functions through the RAGE[65-67].

AGEs and RAGE also play an important role for DAN[68-70]. The expression of AGEs has been demonstrated in the peripheral nerves in DM animals[71] and in the axons and Schwann cells of patients with DM[72]. Increased expression of RAGE in peripheral nerves in DM rats has also been demonstrated[73]. The AGEs-induced changes of proteins could cause structural and functional changes in the peripheral nerves[74]. Modification of major axonal cytoskeletal proteins such as tubulin, neurofilament, and actin by AGEs impairs axonal transport and contributes to the development of atrophy and degeneration of nerve fibers[75,76]. Microvessels in peripheral nerves affected by AGEs may also contribute to the damage of peripheral nerves[77]. Therefore, long-term hyperglycemia induced GI tissue nonenzymatic glycation appears to play an important role in the remodeling of GI wall in DM.

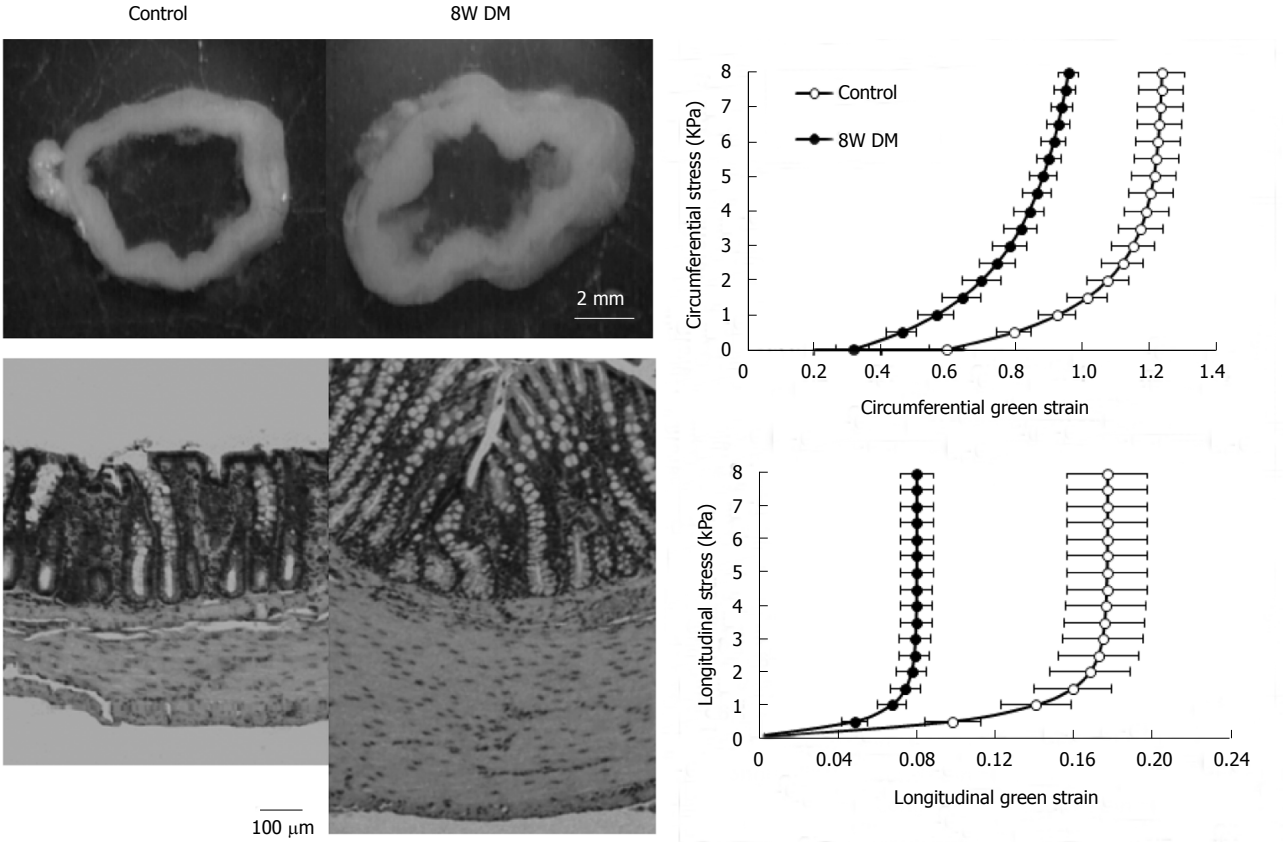

Figure 2 Colonic remodeling in STZ-induced diabetic rats. The top-left figure showed the no-load tissue rings of colon from control (left) and 8W streptozotocin-induced diabetic rats (right). It clearly demonstrated that the wall thickness increased in the diabetic colon. The low-left figure showed micro-photographs of the control (left) and 8 wk diabetic (right) colonic histological sections. It clearly demonstrated that the mucosa and muscle layers in the diabetic colon became much thicker than in the normal colon. The bar is 100 μm. The right figures showed the relation between circumferential (top) and longitudinal (bottom) stress and strain. Both in the circumferential and the longitudinal directions, the stress-strain curves shifted to the left in the 8W diabetic groups compared to those in the control group. Thus, the colon wall stiffness increased in both directions during the development of diabetes. Control: Normal control; 8W DM: 8 wk of diabetes.

DIABETES-INDUCED SENSORY-MOTOR CHANGES IN THE INTESTINE AND COLON

DM-induced GI remodeling likely affects the sensory-motor function through the modification of the mechanical environment and structural basis around the motor and sensory nerves in the wall of intestine and colon. DM-induced increase in wall stiffness can change the tension and stress distribution around the mechanosensitive afferents. DM-induced structural and deformational changes can alter the relative position and response rate of the motor-sensory afferents. Furthermore, DAN involves both the sensory nerve supply to the intestine and colon, the ENS and processing in the central nerve system (CNS). Therefore, it is important to explore DM-induced sensory-motor changes in the intestine and colon and its mechanisms.

Diabetes-induced motor changes in the intestine and colon

Small intestine (Table 3): Both delayed and rapid transit has been demonstrated in DM animal models[78,79]. It has been found in DM rats that the increase in transit time and decrease in intestinal tone are associated with up-regulated cholinergic activity and low-regulated beta-adrenergic receptor activity[80]. Stress-strain analysis of jejunal contractility in response to flow and ramp distension demonstrated that the jejunal contractility was hypersensitive to stimulations after carbachol application[81,82] in type 2 DM rats (Figure 3). However, the force generated per unit of smooth muscle was decreased in the DM rats, and could be partly compensated by hyperplasia and hypertrophy of the smooth muscle[82]. Furthermore, it was demonstrated that the ileal segment from type-1 DM rats was hypersensitive to distension for contraction induction[83]. However, the contraction force produced by smooth muscle was lowest in DM rats. Increased AGE and RAGE expressions were found to be associated with contractility changes in DM rats.

In DM patients, the delay in intestinal transit time has also been demonstrated by using different tests such as breath hydrogen appearance time[84], radiopaque markers[85] and metal-detector[43]. On the contrary, increased intestinal transit time has been found in insulin-dependent DM (IDDM) patients[86]. In patients with long standing IDDM, it has been demonstrated that the duodenal transit is disturbed and the chyme

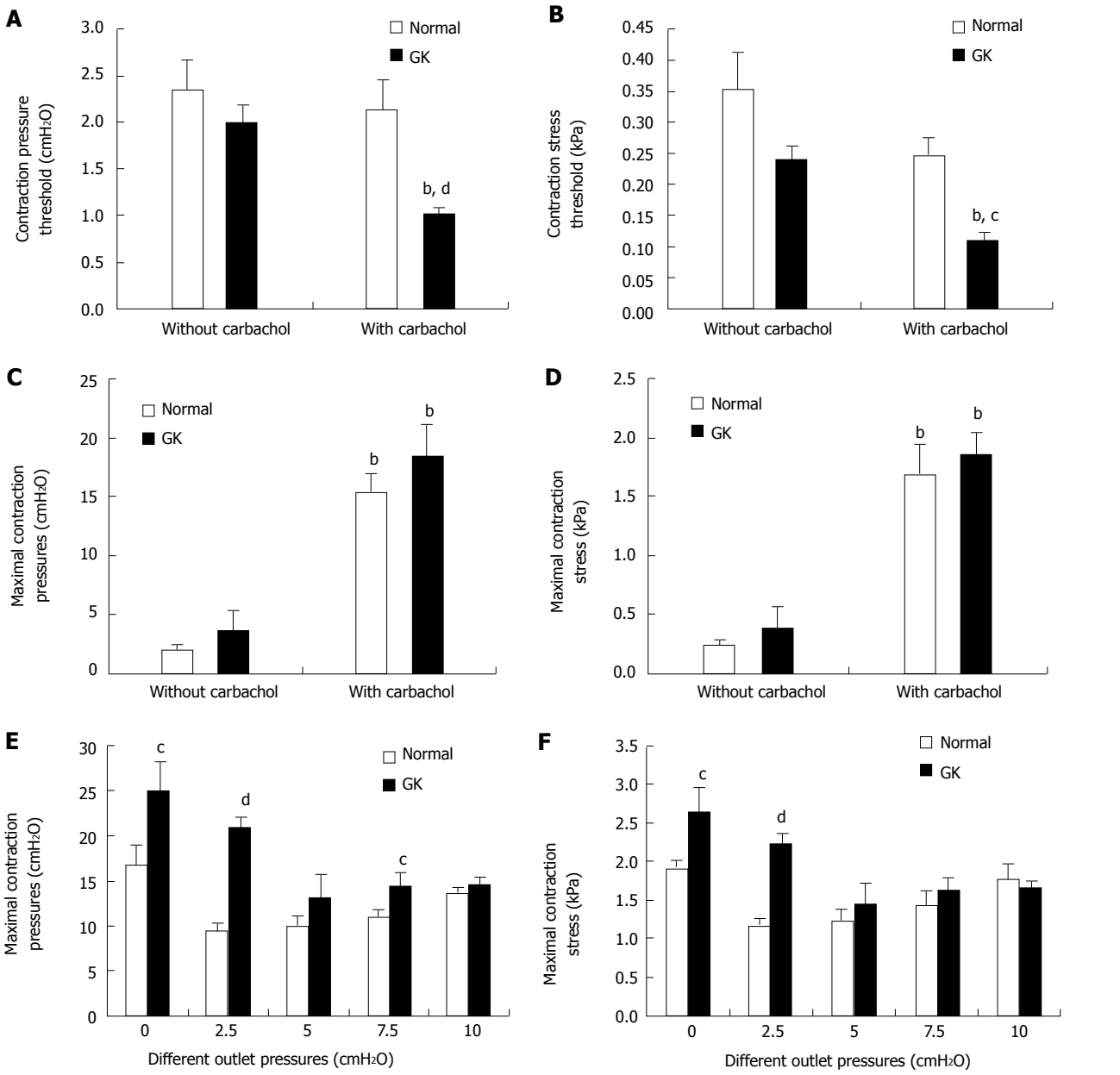

Figure 3 Jejunal contractility in response to flow and ramp distension in type 2 diabetic GK rats after carbachol stimulation. Top figures showed the pressure (A) and circumferential stress (B) at the contraction threshold during ramp distensions. The pressure and stress thresholds were significantly decreased in GK group but not in Normal group after carbachol application (compared with without carbachol application, $^{b}P < 0.01$). Furthermore, the pressure and stress thresholds were significantly smaller in the GK group than in Normal group after carbachol stimulation (compared with Normal group, $^{c}P < 0.05$; $^{d}P < 0.01$). Middle figures showed the maximum contraction pressure (C) and stress (D) during basic contraction. After carbachol application, the maximum contraction pressure and stress significantly increased both for Normal and GK groups (compared with without carbachol application $^{b}P < 0.01$). Bottom figures showed the maximum contraction pressure (E) and stress (F) in the flow-induced contraction after carbachol application. Compared to the Normal group, the maximum contraction pressure and stress were significantly bigger at outlet pressure levels of 0 and 2.5 cmH2O in the GK group ($^{c}P < 0.05$, $^{d}P < 0.01$).

clearance activity is decreased[87]. In one study, it has been reported that about 80% of patients with long-standing DM had abnormal motility of the small intestine[88]. The DM-induced dysmotility can occur either in the postprandial or fasting state[89,90]. In noninsulin-dependent DM (NIDDM) patients with diarrhea and DAN, grossly disordered motility such as migrating motor complex disorders has been reported[89]. Although disorders of postprandial motility in small intestine have been reported in DM patients, the findings are inconsistent[90].

Colon (Table 3): Colonic dysmotility is often seen in DM patients[85,91-97] and animal models[97-103]. DM patients with DAN are expected to have delayed transit in the entire gut, this finding is apparent to some extent in the distal colon but not in the proximal colon[92]. Delayed transit is most frequent in male patients with long-term IDDM where the total colonic transit time is prolonged[93]. Even in type II diabetic patients without clinical presentation of neuropathic symptoms, significant elongation of the transit time has been observed in the lower digestive tracts compared

Table 3 Diabetes mellitus-induced motor and sensory changes of intestine and colon

Changes	Intestine	Colon
Motor	Transit time ↑↓[43,78-80,84,87] Muscle tone ↓[80] Jejunal contractility in response to flow and ramp distension after carbachol application ↑[81] Ileal contractility in response to distension ↑[83] The force generated by the smooth muscle per unit ↓[82,83] Dysmotility DM patients[88,90] Migrating motor complex disorders[89]	Transit time ↑[85,92-99,101,102,106] Contractility ↑↓[104,108,109,111-113] Carbachol induced contractions in muscle ↑↓[100,107,112] Spontaneous contractility ↑↓[100,102] Contraction and relaxation of circular muscle strips from DM were impaired[105]
Sensory	Sensitivity of human duodenum to the combination of mechanical, thermal and electrical stimulations ↓[114] Sensitivity of rat jejunum to the mechanical stimulation ↑	Sensitivity of rat colon to the mechanical stimulation ↑[103,115,116]

DM: Diabetes mellitus.

to control subjects[85]. Jorge *et al*[95] found that at 24 h after ingestion, there was no difference in the number of radiopaque particles in the colon between DM patients and controls. However, at 72 h past ingestion, the mean number of radiopaque particles in the colon was significantly higher in DM patients than in healthy controls. Furthermore, The DM patients with constipation had longer colonic transit times than those without constipation[94,96]. Hyperglycemia could inhibit long and short neural reflexes to modulate colonic motility which may contribute to constipation in DM[104]. The postprandial colonic motility is increased in DM patients with mild constipation but not in DM patients with severe constipation, the later may be due to DAN-induced absence of the postprandial gastrocolonic response[91]. Chandrasekharan *et al*[105] demonstrated that colonic circular muscle strips from DM subjects showed impaired contraction and relaxation responses compared to that of healthy controls. Such changes may be caused by the loss of enteric neurons in the colon due to increased oxidative stress and apoptosis.

Results from animal studies are ambiguous and have shown both delay and enhancement in the colon transit time in DM. Similarly, both reduced and increased colon contractility for whole segment or muscle strips in DM animals are reported. Delayed colonic transit has been found in alloxan-induced DM mice[98], db/db mice[99] and DM rats[101,102]. The prolonged transit time in db/db mice is associated with reduced areas of ICC and the expression of SCF in colon[99]. Insulin-like growth factor 1 (IGF-1) treatment can inhibit the DM-induced colonic smooth muscle cell apoptosis and may be involved in the alleviation of colonic dysmotility in DM rats[102]. However, Domènech *et al*[106] reported that DM RIP-I/hIFNβ transgenic mice showed an enhanced gut transit associated with gut remodeling including neuroplastic changes and overt myenteric neuropathy. In relation to the contractility, however, carbachol-induced and Ems-induced contractions in the colon muscle were significantly reduced in DM mice[107]. Wang *et al*[101] showed that endogenous IGF-1 and SCF protein and their mRNA expressions were significantly reduced in the DM colonic muscle tissues. Kim *et al*[100] demonstrated that spontaneous contractility decreased, carbachol-induced contractility decreased and the number of interstitial cells of Cajal networks was greatly reduced in the proximal colon of DM rats. In addition, the degree of relaxation in response to nitric oxide in the proximal colon of DM rats also appeared to be attenuated. Their results suggest that the decrease of interstitial cells of Cajal network, cholinergic receptors, and neuronal nitric oxide synthase in the proximal colon plays important roles in DM-related dysfunction of colon. Touw *et al*[108] showed that Type 1 DM is associated with decreased depolarization-induced Ca(2+) influx in colonic smooth muscle, leading to attenuated myosin light chain phosphorylation and impaired colonic contractility. Sung *et al*[103] showed that the frequency, not the amplitude, of colonic spontaneous contraction in vitro was significantly decreased in DM rats compared to control rats. However, enhanced contractility of the colon in the DM animals has also been reported[109,110]. The increased contractility is associated with loss and injury to ICC in the submucosa and muscle layers[110]. Yoneda *et al*[111] showed that the colonic peristaltic reflex is enhanced by impairment of enteric nitrergic inhibitory neurons in spontaneous DM rats. Xie *et al*[112] demonstrated that carbachol-induced contractions of distal colonic strips were greater in DM rats in which β-arrestin2 is involved in the increase of distal colonic contraction in DM rats. Chang *et al*[113] indicate that the increased contractions of distal colon in DM rats are partly mediated by the IL-6 receptor pathway.

Diabetes-induced sensory changes in the intestine and colon

Compared with published studies on motor disorders in DM, only few studies have addressed the sensory function of intestine and colon in the DM (Table 3). In relation to the small intestine, it has been demonstrated in a human study that there was an overall hyposensitivity to the combination of all stimulations including mechanical, thermal and electrical stimulations in the duodenum in the DM patients[114]. Furthermore, it

was found that these patients demonstrated a 46% increase in the somatic referred pain areas. This may indicate that central neuronal changes are involved in the sensory changes of gut. Thus, the mechanisms of GI symptoms in long-standing DM patients are likely to involve the interactions between peripheral and central systems[114]. In relation to the colon, Grabauskas *et al*[115] showed that visceromotor responses to colorectal distension were significantly higher in STZ-induced DM rats 8 wk after the induction of DM. Such visceral hypersensitivity is mediated by abnormal IA current resulting from an increased phosphorylation of MAPK and Kv4.2 in dorsal root ganglion neurons. Similarly Hu *et al*[116] has also demonstrated that STZ-induced DM rats had colonic hypersensitivity to mechanical stimulation. The hypersensitivity was associated with an enhanced neuronal excitability of primary sensory neurons that showed an up-regulated expression of voltage-gated sodium channels (VGSCs, *i.e.*, NaV1.7 and NaV1.8 subunits). Visceral hypersensitivity is also demonstrated in a rat model of type 2 DM accompanied by weight loss[103].

Mechanism of sensory-motor function changes

It is important to understand the mechanism behind the DM-induced sensory-motor changes of gut in order to enhance treatment approaches for the DM patient with gut disorders. As mentioned previously, the histomorphological and biomechanical remodeling could alter the baseline of the mechanosensitive afferents activity and the biomechanical environment around the mechanosensitive afferents. Therefore, DM-induced changes of gut structure and biomechanical properties can induce the changes observed in sensory-motor functions. On the other hand, the sensory-motor changes of the gut may reflect the structural and functional changes of peripheral nerve, ENS and the CNS in patients with DM. It seems that the more severe the neuropathy, the greater the likelihood of the involvement in gut sensory-motor disorders is[9].

More than 30% long-standing DM patients have DAN, therefore DAN is the most prevalent DM complication which is also related to other diabetic complications including GI complications[117]. The sensory nerves and ENS can be affected by peripheral DAN[7,118,119]. There is proof that the nerves in different layers of the GI wall undergo DAN changes and that the parasympathetic fibers in the gut are disrupted in DM patients[114]. Furthermore, ICCs are pacemaker cells in the GI tract distributed along the GI wall[120]. In GI tract, ICCs play an important role for the link between the autonomic nervous system, enteric neurons and smooth muscle cells[121]. Animal studies have demonstrated that the number of ICCs is reduced in different parts of GI tract such as the stomach[99,122], small intestine[99,123] and[99,124] colon. Therefore, the mechanisms of DM-induced sensory-motor function changes extensively involve the autonomic nervous system, ENS and ICCs.

Many factors are related to DAN. As mentioned above, the formation and accumulation of AGEs in peripheral nerves are associated with DAN directly by affecting structural and functional proteins and indirectly by activating receptors for AGEs. In addition to the formation of AGEs, the microvascular complications to DM causing neuropathy include other biochemical pathways. For example, DM can induce oxidative stress which is enhanced by AGE formation and polyol pathway activation[125]. Many data have shown that oxidative stress-induced tissue injury is associated to DAN[77]. Experimental and clinical data provide evidence that C-peptide is related to nerve dysfunction in DM since the C-peptide administration by subcutaneous injections seems to ameliorate nerve dysfunction in DM[126]. Human and animal studies have demonstrated that Na^+, K^+-ATPase activity is impaired in the cell membrane of many tissues (see details in review)[127]. Thus, the impairment of Na^+, K^+-ATPase activity also plays an important role in the development of DAN by different pathways[128]. Furthermore, increased polyol pathway in DM has long been regarded as important in DAN[129]. Animal data suggests that glucose shunting through the polyol pathway alters nerve excitability due to the formation of sorbitol[130]. Furthermore, structural alterations of the nerves including thickening of the capillary basal membrane, loss of capillary pericyte coverage and endothelial hyperplasia, all lead to disturbances in capillary flow compromising the exchange of oxygen and glucose[131].

CLINICAL CONSEQUENCES OF DIABETES-INDUCED CHANGES IN INTESTINE AND COLON

DM-induced sensory and motor dysfunction can affect part of or the entire GI tract, therefore, the perceived symptoms may be associated with one or several parts of the GI tract. In the patients of both IDDM and NIDDM, the GI symptoms are very common and can reach 75%[132-138]. Due to the non-specific nature of GI symptoms in DM patients, differential diagnoses should be considered when clinicians deal with GI symptoms. As DM-induced DAN can affect the enteric nerves supplying the small intestine and colon, abnormal motility, secretion, absorption and transportation can occur as possible outcomes. The clinical manifestation of these can be symptoms such as central abdominal pain, bloating, diarrhea, incontinence and constipation. An overrepresentation of celiac disease has been observed in insulin dependent DM patients, as this is a known etiologic factor of severe diarrhea, celiac disease should be excluded when clearing up the matter of general DM diarrhea[139]. Recent evidence indicates that GM is strongly associated with the development of type 1 and type 2 diabetes[97,140,141]. Furthermore, a close relationship between DM and increased risk of colon cancer has been demonstrated in both women and men[142,143]. DM is considered an independent risk factor

for colon and rectal cancer[144].

Diarrhea and diabetes

Chronic diarrhea is a frequent presenting symptom, seen by both general practitioners and gastroenterologists. The differential diagnosis is broad, and diagnostic evaluation may be complicated[145,146]. Diarrhea is an important and often debilitating feature of DM enteropathy occurring in up to 20% of the patients[147]. It has also been reported that chronic diarrhea is more frequent in type I DM patients[148]. The diarrhea is typically painless, may be associated with fecal incontinence and occurs more often at night[149]. Therefore when gastroenterologists are confronted with patients suffering from chronic diarrhea, DM should be considered as a differential diagnosis, especially poorly controlled DM with co-existing DAN.

Many factors may relate to diarrhea in DM patients. These include food composition, intestinal motility disorders, GM changes, excessive loss of bile acids, pancreatic insufficiency and *etc*.[148,150-152]. Both increasing and decreasing GI transit time in DM patients may cause diarrhea. If the transit time become fast, intraluminal contents reaching the caecum will increase[153]. If the transit time is slow, there is a risk of bacterial overgrowth. Therefore, both conditions can potentially induce DM diarrhea[154]. The etiology of DM diarrhea is not fully understood and is most likely multifactorial[4,155], involving reversible and irreversible processes. The diarrhea does not always correlate with the duration of DM or glycemic control, therefore DAN is thought to be a main underlying mechanism[156].

The colon likely plays a secondary or permissive role in patients with steatorrhea which could be caused by pancreatic insufficiency, celiac disease, or bacterial overgrowth[157]. However, the colonic dysfunction may be a primary contributor in DM diarrhea where the steatorrhea is absent. Other causes of diarrhea also need to be excluded, such as infectious diarrhea, celiac disease, bile salt diarrhea, and the concomitant use of drugs that may cause diarrhea such as metformin, GLP-1 receptor agonists, dipeptidyl peptidase 4 inhibitors, proton pump inhibitors, and statins[158] as well as functional diarrhea[159].

Constipation and diabetes

Constipation may be the most common GI complaint in DM patients[4,96,147,157,160-165]. Constipation also represents the most severe symptomatic problem[132]. The severe constipation may clinically present as obvious abdominal distention, severe nausea and vomiting as well as electrolyte disturbances[157]. Long-term and severe constipation may also cause stercoral ulcerations and perforation.

The etiology of DM constipation is not well understood; however, all factors in the DM patients affecting motor-sensory function of the colon are likely associated with constipation[164]. Among these factors, hyperglycemia is suggested to be the most important one. Inadequate glycemic control and consequent DAN have great influence on the sensory and motor functions of the GI tract[105]. DM angiopathy and vascular complications secondary to chronic hyperglycemia can also cause intestinal ischemia and impair nerve and muscle function resulting in DM gastroenteropathy[166]. Hyperglycemia causes apoptosis of enteric neurons and changes in their chemical code, resulting in motility changes[105]. It is well known that long-term hyperglycemia can induce the formation and accumulation of AGEs which play an important role for DAN[68-70]. ICC, together ENS and smooth muscles, play an important role in the regulation of motility[120]. One study demonstrated that a high dietary saturated fat intake is associated with significant increase in the prevalence of constipation in patients with uncontrolled DM[167]. The long term high dietary saturated fat consumption leading to slower GI motility and constipation may be related to gastrocolic reflux by several mechanisms. In addition, other factors such as stress, inflammation and functional changes in relation to DM may also be associated with constipation in DM patients[164].

Gut microbiota modification

The greatest concentration of microorganisms is found in the GI tract, and they consist mostly of bacteria[168]. The GM plays an important role in normal intestinal function and maintenance of the host health[168]. Composition of GM is affected by many factors such as diet, disease state, medications as well as host genetics. Therefore, GM has been associated with immune functions, immune mediated diseases, energy homeostasis and obesity[169,170]. To date, it has become increasingly evident that GM contributes to both type 1 and type 2 DM[140,141,171]. In recent years, many reviews have discussed the impact of GM on the development of obesity and DM[140,171-174]. However, to the best of our knowledge, there are few reviews which discuss how the DM-induced GI changes in turn affect the GM. It is well known that the GM inhabits the gut, therefore the DM-induced intestinal and colonic changes are likely to modify GM composition, in turn, the GM changes may also affect intestinal structure and motility.

As we discussed above in this review, motility disorders are common in diabetic patients[78-97]. There is evidence to suggest that modification of GI transit time can affect the composition of the GM community[175-177]. A close relationship exists between transit time and GM mass[175] and the motility shapes the composition and function of GM[176]. Therefore, the abnormal motility of gut in DM such as decreased or increased transit time can be an independent factor affecting the amount, the composition and the function of GM[178]. The changes of GM may further affect gut function through the brain-gut-axis[179]. The GM can interact with the gut-brain-axis by means of the modulation of afferent sensory nerves to modulate the motility of the intestine[180,181]. The GM can also directly affect the ENS by different molecular pathways[182,183]. Furthermore, the GM can modulate

gut motility by nitric oxide generating pathway[184] and by interacting with the vanilloid receptor on capsaicin-sensitive nerve fibers[185].

The DM-induced intestinal histomorphological changes such as mucosa damage may also be related to GM modification and function. The leaky epithelium presumably alleviates the penetration of bacteria through the intestinal epithelium, initiating a pathologic cascade and disturbing the intestinal immunology, which is a critical element in the development of type 1DM[169]. On the other hand, the GM changes may also affect the integrity of intestinal mucosa[186] and smooth muscle functions[187]. The bidirectional interplay between GM and DM-induced intestinal changes contributes to the pathogenesis of GI disorders in DM.

GLP-1 regulates glucose homeostasis by stimulating the secretion of insulin from pancreatic β-cells[188] and plays important roles in metabolism as well as GI motility[188-190]. In relation to DM, GLP-1 acts as a pharmacological agent with definite therapeutic potential in DM treatment, regulating blood glucose by stimulating insulin secretion from insulin-producing β-cells in a blood-glucose dependent manner and inhibiting glucagon secretion from the glucagon-producing α-cells[191,192]. On other hand, it has been demonstrated that GLP-1 is progressively up-regulated in pancreatic islets during type 2 DM development[193]. More recently, the link between GLP-1/GLP-1 receptor (GLP-1R) expression and GI motility mediated by GM has been investigated[194]. They found that the expression of GLP-1R in myenteric neural cells in the GI tract was suppressed and the GI transit time became shorter in Germ-free (GF) mice after transplantation of GM. Therefore, they suggest that the GM accelerates the GI motility while suppressing the expression of GLP-1R in myenteric neural cells throughout the GI tract. There are also other anti-diabetic agents which act in the GI tract such as alpha-glucosidase inhibitors[195] and GLP-1 receptor agonists[196,197]. It is interesting to notice that alpha-glucosidase inhibitors and GLP-1 receptor agonists also affect the GM[198-200]. Alpha-glucosidase inhibitors such as acarbose treatment has been demonstrated to increase the content of gut Bifidobacterium longum and partially restore the imbalance of GM in patients with type 2 DM[198], and the changes in GM are strongly associated with the levels of various metabolic indicators[200]. In contrast, GLP-1 receptor agonists such as liraglutide seem to modulate the composition of the GM[199]. Other therapeutic agents targeting DM such as metformin[201] and antibiotics[202] also affect the GM. Thus, there is an interplay between drugs used for DM and the GM, however, the exact mechanism of the interaction is complex and needs to be investigated more thoroughly.

Colon cancer and diabetes

Type 2 DM mellitus has been reported to increase the risks of a wide spectrum of cancers including colorectal cancer[142-144,203-206]. Colorectal cancer is a significant health problem; it is one of the most common malignancy of the GI tract[207]. Therefore, understanding the association between DM and the risk of colon cancer is crucial.

Although some studies have reported no overall associations between DM and colon cancer risk[208-211], most studies support the finding of an association between DM and colon cancer. Large prospective studies have demonstrated that DM is associated with an increased risk of colon cancer in people investigated[211-217]. Many meta-analysis studies also support a correlation between DM and increased risk of colon cancer[142-144,218-222]. A population-based cohort study investigating the overall sex- and age-specific risks of colorectal cancer in association with DM was done by Chen *et al*[223]. They showed that DM significantly increased the risk of colorectal cancer, especially in patients aged 45-64 years. A multiethnic Cohort study also found that DM is a risk factor for colorectal cancer[224]. In addition, DM was found to negatively impact the survival outcomes of patients with colon cancer[225].

The mechanisms to explain the association between DM and increased colon cancer risk remain unclear. It has been demonstrated that AGEs and RAGE are up-regulated in the DM GI tract[49,64], and AGEs and RAGE are associated with DM-induced intestinal and colonic histomorphological remodeling[47,51] and DAN[125] which is closely related to motor-sensory disorders[9]. High glucose levels and AGEs increase the proliferation and migration of cultured colon cancer cells[226]. Hyperglycemia and AGEs could also induce oxidative stress and inflammation, which can cause further damage to the cellular components and contribute to malignant cell transformation[227]. Inflammation is a critical component of DM-induced target organ injury and colon cancer initiation and progression[228,229]. The inflammasome regulates the microbiota and the inflammatory response of epithelial cells to the GM[230], and the GM has been shown to be associated with GI malignancy including colonic cancer[231,232]. Recent studies suggest that RAGE signaling plays an important role in colorectal tumor progression[233]. Furthermore, AGEs may promote cancer cell proliferation through the activation of the RAGE signaling[234,235]. Therefore, hyperlipidemia, AGEs, inflammation, extracellular matrix alterations, and altered microbiota may induce GI tissue injury that may favor the development of colonic cancer. It has also been demonstrated that the slower bowel transit time in DM patients could enhance the exposure of the colorectal epithelium to carcinogens such as bile acids, nitrosamines and polycyclic hydrocarbons[236]. The above-mentioned findings show a possible link between DM-induced intestinal mechanophysiological changes and colonic cancer. The potential molecular mechanisms mediating the link between DM and colon-rectal cancer have been reviewed in detail recently[237], however the exact mechanisms need to be explored further.

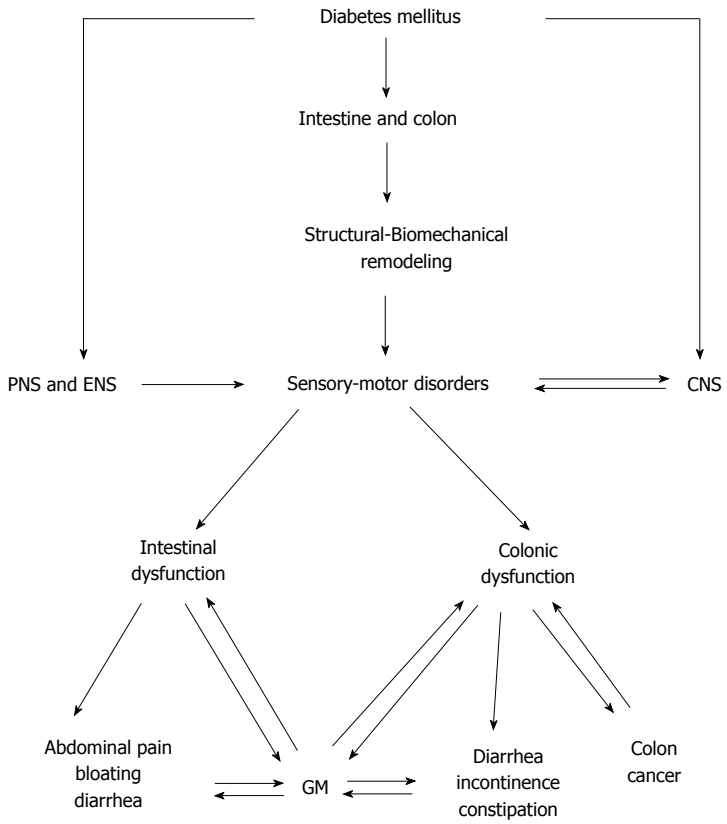

Figure 4 The diagram shows the diabetes mellitus-induced intestinal and colonic changes and clinical consequences. CNS: Central nerve system; PNS: Peripheral nerve system; ENS: Enteric nervous system; GM: Gut microbiota.

Furthermore, other factors as mentioned below are also likely associated with colon cancer. Metabolic syndrome, characterized by abdominal obesity, hyperglycemia, raised blood pressure, elevated triglyceride levels, and low high-density lipoprotein-cholesterol levels, is often seen in diabetic patients and has been reported to be associated with colon cancer[238-241]. As dietary fibers reduce the risk of metabolic syndrome, dietary fibers may have a role in the prevention of colon cancer in patients with type 2 DM[242]. Chronic hyperinsulinemic state and the elevation of insulin-like growth factor-1 levels may play a crucial role in the proliferation of cells and the occurrence of colon cancer[243] by different molecular mechanisms[244]. Elevated insulin receptor protein expression in colonic tumors has also been proposed as a possible biological mechanism for colonic tumorigenesis as *in vivo* studies have shown that insulin receptors contribute to cell transformation[245]. Different treatments of DM may also be related to colon cancer in DM patients. Chronic insulin therapy has been reported to be associated with an increased risk of colorectal adenoma[246] and cancer risk[247] among type 2 DM patients. Sulfonylureas stimulate endogenous insulin secretion and have therefore been suggested to be associated with an increased risk of colon cancer[248], however other reports showed that sulfonylurea use was associated with a lower colon cancer risk in DM patients[249]. Data on potential carcinogenic effects of thiazolidinediones are inconsistent, but most studies have found no increased risk of colon cancer[244,250,251] or even reduced risk of colon cancer. Metformin lowers the amount of circulating insulin and there is evidence on Metformin acting as a protective agent against colon cancer[234,252-254], this may be due to a reduction of the formation of precancerous lesions[255,256]. GLP-1-based therapeutic approaches have also been suggested as potential carcinogenic factors[102]. However, animal studies have shown that GLP-1 receptor activation reduced growth and survival in mouse CT26 colon cancer cells[257] and GLP-1 receptor agonists did not accelerate neoplasia in carcinogen treated mice[258]. A recent study also demonstrated that DM medication in general did not impact cancer recurrence or survival[259].

CONCLUSION

DM-induced intestinal and colon changes are summarized in Figure 4. DM is a chronic disease and is one of the major public health problems worldwide. Disorders of intestine and colon are common in DM. DM is associated with structural changes in the connective tissue matrix and in the muscles in the wall of intestine and colon and further causes biomechanical remodeling. As demonstrated in the text above, many mechanophysiological changes occur in the diabetic intestine and colon such as changed dimensions and changed passive and active tissue properties. Remodeling also occurs in the nerve structure and function. The interplay between these changes is extremely complex and need a scientific base to be explored fully. The changes may to various degrees be part of the mechanisms responsible for the intestinal and colonic

sensory-motor disorders causing a variety of symptoms. The complexity is even more difficult to deal with since the symptoms are associated with changes in the central processing of visceral afferent signals from the gut wall. As DM-induced DAN can affect the enteric nerves supplying the intestine and colon, abnormal motility, secretion, absorption and transportation can occur. This presents clinically as symptoms including central abdominal pain, bloating, diarrhea, incontinence and constipation. The DM-induced structural changes and motility disorders of the intestines are associated with GM changes in DM, on the contrary the GM changes may in turn affect intestinal structure and motility. Furthermore, studies suggest an association between DM and increased risk of colon cancer in both women and men, and the link between DM-induced intestinal mechanophysiological changes and colon cancer need to be explored further. Therefore, an insight into DM-induced intestinal and colonic changes and the clinical consequences is important in order to explore better treatment approaches for the gut disorders in diabetic patients.

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MINIREVIEWS

Effects of glucose-lowering agents on ischemic stroke

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Abstract

Diabetes mellitus (DM) is a major risk factor for cardiovascular events, including ischemic stroke. Moreover, ischemic stroke appears to be more severe in these patients and to be associated with less favorable outcomes. However, strict glycemic control does not appear to reduce the risk of ischemic stroke. On the other hand, newer glucose-lowering agents (glucagon-like peptide 1 receptor agonists and sodium-glucose cotransporter 2 inhibitors) reduced the risk of cardiovascular events in recent randomized, placebo-controlled trials. Semaglutide also reduced the risk of ischemic stroke. These benefits are independent of glucose lowering and might be due to the favorable effects of these agents on body weight and blood pressure. Pioglitazone also reduced the risk of recurrent stroke in patients with insulin resistance or type 2 DM but the unfavorable safety profile limits its use. In contrast, sulfonylureas and dipeptidyl peptidase 4 inhibitors have a neutral effect on cardiovascular morbidity and might be less attractive options in this high-risk population.

Key words: Antidiabetic treatment; Ischemic stroke; Cardiovascular events; Glucose regulation; Neuroprotection

Core tip: Diabetes mellitus is a major risk factor for ischemic stroke. However, strict glycemic control does not appear to reduce the risk of ischemic stroke. On the other hand, glucagon-like peptide 1 receptor agonists and sodium-glucose cotransporter 2 inhibitors reduce the risk of cardiovascular events. These benefits are independent of glucose lowering and might be due to favorable effects on weight and blood pressure. Pioglitazone also reduced the risk of recurrent stroke but the unfavorable safety profile limits its use. Finally, sulfonylureas and dipeptidyl-peptidase-4 inhibitors have neutral effects on cardiovascular morbidity and might be less attractive options.

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INTRODUCTION

Diabetes mellitus (DM) is a major risk factor for cardiovascular events, including ischemic stroke[1,2]. Even pre-diabetes, defined as impaired glucose tolerance or impaired fasting glucose, is associated with increased risk for ischemic stroke[3]. In a case-control study in 32 countries[4], DM accounted for approximately 16% of the population attributable risk for ischemic stroke. Interestingly, among patients with DM, women have higher risk for stroke than men[5].

Type 2 diabetes mellitus (T2DM) is usually initially managed with metformin monotherapy and, if not controlled adequately, a variety of other glucose-lowering agents can be added[6,7]. In the present review, we summarize the existing evidence on the effects of antidiabetic agents on the incidence of ischemic stroke.

EFFECTS OF AGGRESSIVE GLUCOSE LOWERING ON THE RISK OF STROKE

In the United Kingdom Prospective Diabetes Study (UKPDS), metformin reduced the risk of DM-related and all cause mortality in overweight patients with newly diagnosed T2DM[8]. In contrast, in the same study, treatment with sulphonylureas or insulin had no effect on cardiovascular morbidity[9]. Moreover, in patients with long-standing T2DM, 2 large randomized controlled trials (RCT), the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation trial and the Veterans Affairs Diabetes Trial, showed that more *vs* less aggressive glycemic control had no effect on the incidence of cardiovascular events, including nonfatal stroke[10,11]. Moreover, in the Action to Control Cardiovascular Risk in Diabetes trial (n = 10251 patients with T2DM and established cardiovascular disease (CVD) or additional cardiovascular risk factors)[12], intensive glucose lowering reduced the risk of myocardial infarction (MI) by 20% compared with conventional treatment (95%CI: 0.67-0.96; P = 0.015) but all-cause mortality was higher in the former group by 22% (95%CI: 1.01-1.46; P = 0.04) and the incidence of the primary endpoint, including the risk of ischemic stroke, did not differ between the 2 groups. In contrast, multifactorial treatment, *i.e.*, management of blood pressure and dyslipidemia in addition to glucose lowering, reduced cardiovascular morbidity and mortality in patients with long-standing T2DM in the Steno-2 study[13]. However, another study showed that multifactorial treatment may not lower the incidence of cardiovascular events in patients with newly diagnosed T2DM[14].

A meta-analysis of 28 trials (n = 34912 patients with T2DM) showed that intensive *vs* conventional glycemic control reduces the risk of non-fatal MI by 13% (95%CI: 0.77-0.98; P = 0.02) but has no effect on non-fatal stroke[15]. Another meta-analysis of 5 RCTs (n = 33040 patients with T2DM) showed that intensive glucose lowering resulted in a 17% reduction in non-fatal MI (95%CI: 0.75-0.93) but did not affect the incidence of stroke[16]. Therefore, aggressive glucose lowering treatment does not appear to affect the risk of ischemic stroke.

GLUCOSE-LOWERING AGENTS: EFFICACY AND SAFETY

Metformin

Metformin lowers HBA_{1c} levels by approximately 1.0%-1.5% and is generally well-tolerated[6,7]. The most frequent side effects are from the gastrointestinal system whereas the most severe adverse event, lactic acidosis, is extremely rare[6]. Interestingly, metformin reduced the risk of new-onset T2DM in obese patients[17] (Table 1).

Sulfonylureas

Sulfonylureas are also potent glucose-lowering agents and are inexpensive but have low rates of adherence[18] and carry substantial risks of hypoglycemia[6,19] and weight gain[6]. In addition, when added to metformin, glimepiride was less effective than exenatide and liraglutide[19,20].

Thiazolidinediones

Thiazolidinediones have similar potency with metformin and sulfonylureas[6]. In obese patients with prediabetes, rosiglitazone reduced the incidence of T2DM[21,22]. However, the safety profile of these agents is suboptimal. Rosiglitazone appears to increase the risk of MI[23-25], although in a reevaluation of the Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes trial, the risk for first fatal and nonfatal MI was similar during treatment with rosiglitazone and sulfonylurea/metformin [hazard ratio (HR) = 1.13, 95%CI: 0.80-1.59][26]. Both rosiglitazone and pioglitazone are also associated with weight gain, edema, heart failure, bone fractures and urinary bladder cancer[27-31], although another systematic review showed no difference in side effects between pioglitazone and placebo[32].

Dipeptidyl peptidase 4 inhibitors

Dipeptidyl peptidase 4 (DPP-4) inhibitors have moderate glucose-lowering efficacy and relatively high cost but do not increase the risk for hypoglycemia and do not affect weight[6]. In RCTs and in meta-analyses, sitagliptin, vildagliptin and alogliptin had a neutral effect on cardiovascular events[33-36]. In contrast, saxagliptin increased the risk of hospitalization for heart failure but did not affect the incidence of other cardiovascular events[37-39]. Saxagliptin was also evaluated in elderly

Table 1 Effects of antidiabetic agents on glucose levels, other cardiovascular risk factors and ischemic stroke

Agent	Glucose-lowering efficacy	Other favorable effects	Effect on ischemic stroke
Metformin	High	Weight loss	Decrease
Sulfonylureas	High	(-)	No effect
Thiazolidinediones	High	Reduction in	Might reduce
Pioglitazone		triglyceride levels	the risk of recurrent stroke
DPP-4 inhibitors	Moderate	None	No effect
(1) Alogliptin, saxagliptin, sitagliptin			Reduction (?)
(2) Linagliptin			
GLP-1 agonists	High	Weight loss and blood pressure reduction	No effect
(1) Liraglutide, lixisenatide			Reduction
(2) Semaglutide			
SGLT-2 inhibitors	Moderate	Weight loss and blood pressure reduction	No effect
Empagliflozin			

DPP: Dipeptidyl peptidase; GLP: Glucagon-like peptide; SGLT: Sodium-glucose cotransporter.

patients and was found to have similar safety compared with younger patients[37]. Similar findings were reported for linagliptin[40]. Trelagliptin, a once-weekly DPP-4 inhibitor, was shown to have similar efficacy with daily alogliptin in Japanese patients with T2DM[41]. SYR-472, another once-weekly DPP-4 inhibitor, also appeared to be safe and effective in a phase 2 trial[42].

Glucagon-like peptide 1 receptor agonists

Glucagon-like peptide 1 (GLP-1) receptor agonists are potent glucose-lowering agents, reduce body weight and blood pressure but are expensive and have frequent gastrointestinal side effects[6]. In patients inadequately controlled with metformin monotherapy, adding liraglutide was more effective than adding sitagliptin[43]. More recently, once-weekly preparations of GLP-1 receptor agonists have been developed. Once-weekly exenatide lowered HbA_{1c} levels more than twice-daily exenatide[44] and more than pioglitazone or sitagliptin[45]. However, once-weekly exenatide was less effective than liraglutide[46]. Liraglutide was also more potent than once-weekly albiglutide[47]. On the other hand, once-weekly dulaglutide had similar efficacy with liraglutide[48]. Interestingly, treatment with semaglutide increased the risk of retinopathy (HR = 1.76, 95%CI: 1.11-2.78; P = 0.02)[49].

Sodium-glucose cotransporter 2 inhibitors

Sodium-glucose cotransporter 2 (SGLT-2) inhibitors are a relatively new class of glucose-lowering agents with moderate glucose lowering efficacy[6,7]. They appear to be as effective as sulfonylureas but do not increase the risk of hypoglycemia and induce weight loss and reduce blood pressure[50-53]. However, they are associated with genitourinary infections and diabetic ketoacidosis[50-54]. In a recent RCT, empagliflozin delayed the progression of chronic kidney disease[53]. Empaglifozin also reduced the risk of heart failure[54] and cardiovascular mortality[55].

α-glucosidase inhibitors

α-glucosidase inhibitors are rarely used in the management of patients with T2DM due to moderate efficacy and poor tolerability because of gastrointestinal side effects[6]. On the other hand, voglibose reduced the incidence of T2DM in Japanese patients with impaired glucose tolerance[56].

Insulin

Insulin is the most potent glucose-lowering agent[6]. However, its high cost, risk of hypoglycemia and weight gain represent substantial barriers to its use[57,58].

EFFECTS OF GLUCOSE-LOWERING AGENTS ON ISCHEMIC STROKE

Metformin

In UKPDS, administration of metformin to overweight patients with newly diagnosed T2DM reduced the risk of ischemic stroke more than treatment with sulfonylureas (chlorpropamide or glibenclamide) or insulin (P = 0.032)[8].

Sulfonylureas

In the UKPDS, treatment with chlorpropamide or glibenclamide had no effect on the risk of ischemic stroke. Of note, the relative risk (RR) for non-fatal and fatal stroke in patients who received these agents *vs* conventional treatment was 1.07 (95%CI: 0.68-1.69) and 1.17 (95%CI: 0.54-2.54), respectively, indicating a negative trend for the effects of sulfonylureas[9]. More recently, in a small, multicenter, randomized, double-blind study in 304 Chinese patients with T2DM and established coronary heart disease, metformin reduced the combined endpoint (nonfatal MI, nonfatal stroke, revascularization, cardiovascular and all-cause death) more than glipizide after a median follow-up of 5 years (HR = 0.54, 95%CI: 0.30-0.90; P = 0.026)[59]. Moreover, glimepiride had a less favorable effect than pioglitazone on carotid intima media thickness[60], a marker of subclinical atherosclerosis and a risk factor for ischemic stroke[60]. A systematic review which compared the impact of sulfonylureas on mortality[61], showed that gliclazide and glimepiride were associated with lower rates of cardiovascular and all cause mortality than other members of the class.

Insulin

In the UKPDS, treatment with insulin had no effect on the risk of ischemic stroke[9]. There is no other RCT that evaluated the effects of insulin on the risk of ischemic stroke in patients with T2DM.

Thiazolidinediones

In the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROACTIVE), 5238 patients with T2DM and established CVD were assigned to receive pioglitazone or placebo for 34.5 mo[62]. The incidence of the primary endpoint (all-cause mortality, nonfatal MI, stroke, acute coronary syndrome, endovascular or surgical intervention in the coronary or leg arteries, and amputation above the ankle) did not differ between the 2 groups but the rates of the main secondary endpoint (all-cause mortality, non-fatal MI, stroke) were 16% lower in the pioglitazone arm (95%CI: 0.72-0.98; *P* = 0.027)[62]. Pioglitazone did not reduce the risk of ischemic stroke in the total study population[62] but reduced the risk of recurrent stroke by 47% in the small subgroup of patients (*n* = 984) with a history of ischemic stroke or transient ischemic attack (TIA)[63].

Recently, pioglitazone was also shown to lower the risk of cardiovascular events in patients with insulin resistance and a history of ischemic stroke or TIA. In the Insulin Resistance Intervention after Stroke (IRIS) trial, 3876 patients were randomized to receive pioglitazone or placebo. After a mean follow-up of 4.8 years, the primary outcome (stroke or MI) occurred in 9.0% of patients in the pioglitazone group and in 11.8% of patients in the placebo group (HR = 0.76, 95%CI: 0.62-0.93; *P* = 0.007)[31]. However, the risk of ischemic stroke did not differ between the 2 groups[31].

In a meta-analysis of 19 trials (*n* = 16390), death, MI or stroke occurred in 4.4% of patients receiving pioglitazone and 5.7% receiving control therapy (HR 0.82, 95%CI: 0.72-0.94; *P* = 0.005). Individual components of the primary end point, including stroke, were all reduced to a similar magnitude with pioglitazone treatment, with HRs ranging from 0.80 to 0.92[29]. In another metanalysis of 3 studies in patients with a history of stroke or TIA, pioglitazone reduced the risk of recurrent stroke by 48% (95%CI: 0.34-0.80)[32].

Aleglitazar is a dual agonist of peroxisome proliferator-activated receptors with insulin-sensitizing and glucose-lowering actions and favorable effects on the lipid profile but showed no effect on cardiovascular morbidity in patients with T2DM and a recent acute coronary syndrome and also increased the risk for gastrointestinal hemorrhage and renal dysfunction[64].

DPP-4 inhibitors

In 3 recently published RCTs, the Examination of Cardiovascular Outcomes with Alogliptin *vs* Standard of Care (EXAMINE) study, the Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus - Thrombolysis in Myocardial Infarction (SAVOR-TIMI 53) trial and the Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS), alogliptin, saxagliptin and sitagliptin had no effect on the incidence of ischemic stroke compared with placebo in patients with T2DM and established CVD or additional cardiovascular risk factors[33,35,38]. Moreover, the difference in HbA_{1c} between patients treated with DPP-4 inhibitors and the placebo group were very small (0.20-0.36)[33,35,38]. Another DPP-4 inhibitor, linagliptin, reduced the incidence of cardiovascular events compared with glimepiride in a RCT. This result was mainly attributed to a lower number of non-fatal strokes in patients treated with linagliptin compared with those who received glimepiride (RR = 0.27, 95%CI: 0.08-0.97; *P* = 0.0315)[65]. Of note, a study in mice showed that linagliptin-mediated neuroprotection is glucose-independent and likely involves GLP-1 activation[66].

GLP-1 receptor agonists

In the recently published Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial, 9340 patients with T2DM and established CVD, chronic heart failure, chronic kidney disease or additional cardiovascular risk factors were randomized to receive liraglutide or placebo. After a median follow-up of 3.8 years, liraglutide reduced the incidence of the primary composite outcome (death from cardiovascular causes, nonfatal MI or stroke) by 13% compared with placebo (95%CI: 0.78-0.97)[67]. However, the risk of ischemic stroke did not differ between the 2 groups[67]. In another recent study, the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with type 2 diabetes (SUSTAIN-6), 3297 patients with similar characteristics with the LEADER trial were randomized to receive the once-weekly GLP-1 receptor agonist semaglutide or placebo for 104 wk[49]. Semaglutide reduced the risk of the primary endpoint (death from cardiovascular causes, nonfatal MI or stroke) by 26% compared with placebo (95%CI: 0.58-0.95)[49]. In addition, the risk of ischemic stroke was decreased by 39% in patients who received semaglutide (95%CI: 0.38-0.99; *P* = 0.04)[49]. Notably, in both the LEADER and SUSTAIN-6 trials, the difference in HbA_{1c} levels between the GLP-1 and placebo groups was small and the reduction in cardiovascular event rates appeared to be mostly due to the reduction in body weight and blood pressure in the former group[49,67]. In contrast, another daily GLP-1 receptor agonist, lixisenatide, had no effect on cardiovascular morbidity, including ischemic stroke, in another recent placebo-controlled, randomized trial, the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA) trial (*n* = 6068 patients with T2DM and a recent acute coronary syndrome)[68]. It is unclear whether this negative effect was due to the different study population (*i.e.*, patients with acute coronary syndrome in ELIXA *vs* patients with stable CVD in LEADER and SUSTAIN-6) or whether it suggests that lixisenatide is less effective than liraglutide and semaglutide in preventing cardiovascular events. In a meta-analysis of 9 trials (*n* = 5107), albiglutide also had no effect on cardiovascular events compared with placebo or active treatment (glimepiride, insulin glargine, insulin lispro, liraglutide, pioglitazone, or

sitagliptin) but very few events occurred (n = 116)[69].

SGLT-2 inhibitors

In the recently published *EMPA-REG* OUTCOME trial, 7020 patients with T2DM were randomly assigned to receive 10 mg or 25 mg of empagliflozin or placebo[55]. After a median follow-up period of 3.1 years, the primary composite outcome (death from cardiovascular causes, nonfatal MI, nonfatal stroke) occurred in 10.5% in the pooled empagliflozin group and in 12.1% in the placebo group (HR = 0.86, 95%CI: 0.74-0.99; P = 0.04)[55]. However, rates of ischemic stroke were numerically higher in patients treated with empagliflozin, although TIAs were numerically lower and fatal and recurrent strokes were not increased[55]. Similar to the studies with GLP-1 receptor agonists, the difference in HbA_{1c} levels between empagliflozin and placebo was small, especially after 94 wk (0.24%-0.36%). On the contrary, reductions during first 12 wk were greater (0.54%-0.60%). The reduction in cardiovascular death rates appeared to be mostly due to the reduction in body weight, blood pressure and possibly a diuretic effect of empagliflozin in patients with heart failure[55] suggesting that the effects on ischemic stroke were independent of glucose lowering.

GLUCOSE-LOWERING AGENTS AND NEUROPROTECTION

T2DM is associated with more severe stroke and less favorable outcome[70-72]. Preliminary data suggest that glucose-lowering treatment might alleviate the severity of stroke at admission to the hospital and might improve the functional outcome of these patients[73]. In an early retrospective study, patients who were treated with sulfonylureas prior to stroke and continued to receive them during hospitalization had more favorable functional outcome at discharge[74]. In a prospective study, patients who were on sulfonylureas, metformin or insulin prior to stroke had less severe stroke at admission than those who were not on glucose-lowering treatment[75]. Stroke severity and outcome did not differ between these 3 classes of antidiabetic agents[75]. A small retrospective study also suggested that pioglitazone enhances functional recovery in patients with stroke[76]. Finally, it was also recently reported that treatment with DPP-4 inhibitors prior to ischemic stroke improves the functional outcome at discharge and reduces in-hospital mortality[77]. Linagliptin, a DPP-4 inhibitor, might exert neuroprotective actions[67] and its effect on cognition is currently being investigated in the CAROLINA and CARMELINA trials[78,79].

CONCLUSION

Even though T2DM is a major risk factor for ischemic stroke, strict glycemic control does not appear to reduce cardiovascular morbidity and mortality in these patients compared with conventional treatment. On the other hand, newer glucose-lowering agents, particularly GLP-1 receptor agonists (liraglutide and semaglutide) and empaglifozin, a SGLT-2 inhibitor, appear to reduce the risk of cardiovascular events. Moreover, semaglutide is the only agent that reduced the risk of ischemic stroke in a placebo-controlled trial, although it increased the retinopathy risk. Empaglifozin, on the contrary, might increase the incidence of stroke. It is unclear whether these benefits represent a class effect or are compound-specific. Pioglitazone also appears to reduce the risk of recurrent stroke in patients with prediabetes and established T2DM. On the other hand, sulfonylureas and DPP-4 inhibitors have a neutral effect on ischemic stroke. Basic research showed encouraging results regarding the effects of linagliptin, a DPP-4 inhibitor, on stroke risk, and RCTs evaluating its role in patients with T2DM are ongoing.

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MINIREVIEWS

Treatment of type 2 diabetes mellitus in the elderly

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Abstract

The prevalence of type 2 diabetes is expected to increase gradually with the prolongation of population aging and life expectancy. In addition to macrovascular and microvascular complications of elderly patients of diabetes mellitus, geriatric syndromes such as cognitive impairment, depression, urinary incontinence, falling and polypharmacy are also accompanied by aging. Individual functional status in the elderly shows heterogeneity so that in these patients, there are many unanswered questions about the management of diabetes treatment. The goals of diabetes treatment in elderly patients include hyperglycemia and risk factors, as in younger patients. comorbid diseases and functional limitations of individuals should be taken into consideration when setting treatment targets. Thus, treatment should be individualized. In the treatment of diabetes in vulnerable elderly patients, hypoglycemia, hypotension, and drug interactions due to multiple drug use should be avoided. Since it also affects the ability to self-care in these patients, management of other concurrent medical conditions is also important.

Key words: Diabetes mellitus; Oral antidiabetic drugs; Insulin; Elderly

Core tip: Diabetes mellitus (DM) is one of the most common lifelong chronic diseases in the world and its ratio is increasing by aging population. Elderly patients with type 2 DM have an increased risk for coronary heart disease, stroke and vascular diseases. While determining the treatment target and treatment options in elderly individuals, the functional capacity of the individual, comorbid diseases and treatment compliance should be evaluated together.

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INTRODUCTION

The prevalence of type 2 diabetes is expected to increase gradually with the prolongation of population

aging and life expectancy. In addition to macrovascular and microvascular complications of elderly patients of diabetes mellitus (DM), geriatric syndromes such as cognitive impairment, depression, urinary incontinence, falling, polypharmacy and sarcopenia are also accompanied by aging[1]. Sarcopenia is characterised by a progressive decline in skeletal muscle mass and that is the reason for low muscle strength and impaired physical performance[2]. Elderly (adults over age 65 years) individuals with type 2 DM have a great risk for sarcopenia and physical disability[3]. The mechanism responsible for loss of musclein type 2 DM is uncertain. Changes in skeletal muscle protein turnover may be involved in such alterations in type 2 DM and it can play an essential rol in this pathogenesis[4,5]. There is also a small amount of studies involving elderly diabetic patients, one of the major reason for that is individual functional status in the elderly shows heterogeneity. The physiological changes that develop with aging make it more difficult than studies for to the young age group. As a result, there are many unanswered questions about the management of diabetes treatment in elderly patients. The "patient-centered" treatment regime in the geriatric age group is gaining importance for this reason.

Incidence

Type 2 diabetes is a common health care problem on modern world and it is increasing day by day with the prolongation of life span. In a study conducted in the United States, it was found that the prevalence of type 2 diabetes increased from 16% to 23% between 1995 and 2004[6]. According to the current data, in the United States, among adults over 65 years of age 22% to 33% of them are diagnosed with diabetes. It is predictable that the incidence of diabetes could double in the next 20 years. According to another projection; It is estimated that this increase will be about 4.5 times between 2005 and 2050 in individuals aged 65 and over[1].

Diabetic characteristics in the elderly population

Glucose intolerance increases progressively by aging and the characteristic feature of diabetes in elderly patients is especially postprandial hyperglycemia. Decrease in beta-cell-compensating capacity with advancing age, leads to insulin resistance and it appears as a postprandial hyperglycaemia in the elderly[7]. Therefore, the prevalence varies according to the tests used during diagnosis on elderly patients. One third of the individuals who are tested with A1C or fasting plasma glucose (FPG) are cannot get a diagnosis[8].

The incidence of DM increases with aging. As a result, adults may be diagnosed incidentally after the age of 65, or may have had a diabetes diagnosis in middle age or earlier onset. Having different demographic and clinical characteristics of these two groups may cause confusion caused by the setting of the general treatment recommendations. Age-related DM is characterized by lower A1C and the use of less insulin, with frequent occurrence in non-Hispanic whites. In comparison adults with diabetes diagnosed in middle age, the retinopathy story is more prominent in late-onset diabetic cases, and interestingly there is no difference in prevalence of cardiovascular disease (CVD) or peripheral neuropathy according to age at onset[9]. In diabetic adults increased development risk of lower extremity amputation, myocardial infarction (MI), impaired vision and end-stage renal disease. Patients over 75 years of age have a higher risk of developing multiple complications than the age group of 65-74[10].

Older adults are at higher risk for developing type 2 diabetes because of the combined effects of increased insulin resistance and pancreatic islet dysfunction.

Economic burden

The burden of treatment of older diabetic patients on country economy is quite high. According to the analysis made in the United States in 2010; More than 14 million patients whose age 65 and over are hospitalized annually and approximately one-third of them are diabetic[11]. Again according to United States data, about $245 million is spent yearly on diabetes patients, of which $176 million is direct medical costs, while $69 million is the loss of production and mortality[12]. In addition, 59% portion of the annual treatment costs have been made for the elderly diabetic individuals. Most of the expenditures are caused by hospital admissions, home care and prescripted drugs. These extreme expenditures vary from about $23900 to $40900 per person, depending on gender and age[13].

TREATMENT TARGETS

The goals of diabetes treatment in elderly patients include hyperglycemia and risk factors, as in younger patients. However, the elderly patient group has a heterogeneous structure, some of them are self-caring independently, while the others need care in a nursing home. For this reason, comorbid diseases and functional limitations of individuals should be taken into consideration when setting treatment targets. Thus, treatment should be individualized.

There is limited number of studies investigating the effect of glucose-lowering therapy on cardiovascular complications and mortality. For the reason that the elderly diabetic patient population is not included in clinical trials, we do not have sufficient data on glucose control.

In the United Kingdom Prospective Diabetes Study (UKPDS), in which the effect of glycemic control on microvascular complications is examined, new diagnoses of middle-aged diabetic patients were taken and > 65 years of age were excluded from the study[14]. At the end of the study, there was a statistically significant decrease in both mortality and cardiovascular events in the group ensured early glycemic control. Also observed that microvascular beneficial effects continue after the

study.

After the UKPDS results were published, three important randomized controlled trials (ACCORD trial, Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) trial, and Veterans Affairs Diabetes Trial (VADT) are planned. These studies are designed to examine the effect of glycemic control on CVD events in middle-aged and older patients with type 2 diabetes. The ACCORD trial was terminated approximately 3 years after due to deaths onthe strict glycemic control group[15]. MI, stroke and cardiovascular death were not significantly reduced in primary combined outcomes. In subgroup analyzes, hypoglycemia and other side effects were more commonly detected in elderly participants. In the ADVANCE trial, there was no increased risk of mortality in the strict glucose control group after 5 years of follow-up. When study data were evaluated, there was no statistically significant decrease in cardiovascular risks in the group receiving intensive treatment, however, a significant decrease in the incidence of nephropathy was found[16]. In the VADT trial, there was no statistically significant effect on major cardiovascular events or mortality in the intensive glucose control group after 5 years of follow-up, as well as there wasn't any reversible effect on albuminuria progression. In the intensive group, beneficial effects on mortality were observed in 15 year follow-ups, but higher mortality was found in this group over 20 years[17].

These studies are important in assessing the benefits and risks of strict glycemic control in elderly patients. For this reason, when glycemic control targets are determined, treatment should be individualized considering the life expectancy as well as the chronological age of the patient.

In the treatment of diabetes in vulnerable elderly patients, hypoglycemia, hypotension, and drug interactions due to multiple drug use should be avoided. Since it also affects the ability to self-care in these patients, management of other concurrent medical conditions is also important.

Glycemic targets

In elderly patients receiving medication, there are few data that address the most appropriate glycemic targets. The goals to be determined in the management of glycemic control and risk factors should be based on both the general health status and the predicted life span of the individual. A proper target for A1C in elderly patients with a life expectancy of more than 10 years may vary according to the above factors, the risks of patient-specific hypoglycemia and compliance with treatment regimens[18].

Despite the lack of long-term clinical trials with elderly individuals, patients with life expectancy more than 10 years and drug treated the A1C target should be < 7.5% (58.5 mmol/mol).

Drug treated elderly adults with medically-functional comorbidities and have less than 10 years of life expectancy should have a slightly higher glycemic target [A1C $\leqslant$ 8.0, fasting and pre-prandial glucose should be between 160 and 170 mg/dL (8.9 to 9.4 mmol/L)].

Individualized targets for older adults may be even higher (A1C < 8.5%). The aim of the treatment is to protect the quality of life, prevent hypoglycemia and related complications. Eight point five percent for A1C value and 200 mg/dL for average glucose (11.1 mmol L) should be targeted.

These targets are consistent with the American Geriatrics Association, the American Diabetes Association, the International Diabetes Federation and the European Diabetes Working Group.

In addition, when A1C levels are assessed in elderly patients, accompanying diseases or metabolic conditions should be considered. These include anemia, diseases affecting the erythrocyte life, chronic kidney disease, chronic liver disease, recent blood transfusion or erythropoietin infusion, acute infections and hospitalization. Initial therapy in elderly patients, the same as in younger patients; regulation of nutrition, physical activity, improvement on metabolic control and prevention from complications.

Lifestyle changes

Counseling should be provided on all elderly diabetic patient lifestyle changes (exercise, diet, behavioral changes, and weight loss in patients who need it). In elderly diabetic group response to the lifestyle changes (low fat diet and 150 min/wk exercise) were found to be higher than the young diabetic age group according to the diabetes protection program (DPP)[19].

Physical activity: Elderly diabetic patients should be guided to activities according to their functional capacities. Prior to physical activity, high-risk, symptomatic individuals with coronary artery disease should be evaluated with electrocardiograms and/or cardiac tests. Functionally independent individuals are offered a moderate aerobic activity of at least 5 d for 30 min each week. Except this, patients with high risk of falling should be directed to physiotherapists for balance and muscle strengthening exercises before workout.

Medical nutrition therapy: All elderly diabetic patients should be given medical nutrition education and treatment should be adjusted to their individual needs. When preparing the eating plan, age-related person-specific differences (deterioration in taste, additional illnesses, dietary restrictions, impaired gastrointestinal function, reduced ability to shop, and reduced food preparation capacity) must be considered.

Medical therapy

In elderly diabetic patients, lifestyle changes is recommended with metformin for treatment, primarily because of the risk of hypoglycemia, unless there is a contraindication (renal failure and unstable/acute heart failure)[20]. However, patients with comorbid disease,

multiple drug use, or HbA1c levels close to target levels should be monitored for 3-6 mo with lifestyle changes before initiation of metformin therapy.

At the time of diagnosis patients whose HbA1c level was > 9% (74.9 mmol/mol), FPG level was > 250 mg/dL (13.9 mmol/L), randomly observed glucose value > 300 mg/dL (16.7 mmol /L) or who have ketonuria insulin should be selected as initial theraphy.

There is a small amount of research on the use of medication in elderly patients. All hypoglycemic drugs and insulins can be safely used in elderly patients, with some restrictions. In general, those with low risk of hypoglycemia should be preferred as oral or injected agents.

Pharmacological treatment should be regulated according to the person's abilities and comorbodities. Elderly patients should be treated with the principle of "start low and go slow". Oral antidiabetic drugs and insulin are used in the treatment of diabetes in the elderly.

INSULIN SENSITIZER DRUGS

Metformin

Metformin decreases hepatic glucose production by inhibiting gluconeogenesis in the liver. In many elderly patients, metformin is chosen as the initial treatment. Low cost, positive effects on CVDs, low risk of hypoglycemia the anti-aging effects makes metformin an attractive choice for elderly patients[21]. The most important restricting factor of metformin treatment is glomerular filtration rate (GFR) and treatment can be started if GFR of the patient is > 30 mL/min. It is recommended to use a full dose in patients with GFR > 60 mL/min and a half dose (1000 mg/d) in patients with GFR 30-60 mL/min. Two other factors that limit metformin treatment in elderly patients are weight loss and gastrointestinal side effects. However, the treatment may be started at 500 mg/d and the gastrointestinal side effects may be minimized by slowly increasing the dose within weeks.

Elderly patients should also be used metformin cautiously as it may cause renal dysfunction or lactic acidosis. Patients using metformin should be warned for stopping medication if the use of iodine-containing contrast media is needed for any reason. In addition, evaluation of renal function tests every 3 to 6 mo is necessary.

Thiazolidinediones

Thiazolidinediones are peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist drugs and these drugs regulate the transcription of genes that respond with PPAR activation[22]. These drugs are effective in increasing insulin sensitivity in peripheral tissues, primarily skeletal muscles. It is the group of drugs that can be preferred in the elderly who are not using the insulin treatment and that the sulfonylurea treatment is contraindicated. This drug group being able to be preferred in patients with impaired renal function, as well as low risk of hypoglycemia and has the greatest advantage to be well tolerated in elderly patients. Peripheral edema may develop in 4%-5% of patients during treatment, and is contraindicated in patients with class Ⅲ-Ⅳ congestive heart disease[23]. In addition to these, the most important disadvantages are: It can exacerbate ophthalmopathy, increase the risk of bone fracture, increase weight in combination with insulin therapy, cardiovascular events (fatal and nonfatal MI) and increase in bladder cancer. Patients with active bladder cancer or history, defining macroscopic hematuria or having complaints of hematuria, dysuria, pollakuria, waist and back pain during thiazolidinedione usage should be screened for bladder cancer before the onset of treatment[24]. Because of the disadvantages of them (congestive heart failure, risk for falls or fractures), their use is not recommended on elderly patients, however for the selected patients dosage should be started at the lowest dose and the duration of treatment should be kept as short as possible[25].

INSULIN RELEASING (SECRATOGOGUE) DRUGS

Sulfonylureas

Short-acting sulfonylureas (*e.g.*, glipizide) are recommended as initial therapy in patients with metformin therapy contraindicated or unable to tolerate it. Side effects such as hypoglycemia and weight gain should be considered in addition to glucose lowering effects, easy availability and low cost in the selection of sulfonylureas (SU). Glipizide, glyburide (glibenclamide), gliclazide and glimepiride are 2nd generation SUs and are more potent than 1st generation SU and have less side effects. Glyburide (glibenclamide) and glimepiride have a long half-life and are used in a single daily dose and reduce fasting glucose by inhibiting night hepatic glucose output and cause elevated risk of hypoglycemia[26]. The risk of hypoglycemia is increased especially in patients with renal insufficiency, elderly patients, multiple drug users, patients with dementia, heart failure and long-term diabetes[27].

In elderly patients, sulfonylurea-related hypoglycemia is variable. In general, from diabetic patients in hospital, hypoglycemia was more common in patients aged > 75 years, compared to groups aged 65-74 years. Admission to the hospital with the cause of severe hypoglycemia in the elderly is associated with greater health problems than admission with hyperglycemia, which suggests to have opportunities to improve health through rational drug selection in the elderly with diabetes[28]. Short-acting ones such as glipizide should be preferred if the SU treatment is planned to be given in the elderly. The initial regimen of glipizide 2.5 mg should be regulated as half an hour before breakfast, and if sufficient glycemic control

cannot be achieved within 2-4 wk, the dosage should be increased to 5-10 mg.

Meglitinides

Similar to sulphonylureas, act on pancreatic beta cells to increase the 1st phase of insulin secretion through ATP-dependent potassium channels through different receptors. The blood glucose lowering effect with fast onset and a short duration[29]. Especially the effects on postprandial hyperglycaemia are distinctive, so they are used just before meals. Use of just before each meal can cause discontinuation in the treatment of older adults with cognitive pathology[30]. The most important side effects, though not as obvious as SUs, are hypoglycemia and weight gain. The risk of hypoglycemia is more pronounced in older adults, especially those who skip meals. Repaglinide is metabolised from the liver, so it can be safely used in the elderly with renal insufficiency without need for dose adjustment. It is a good option as initial therapy in older patients with chronic renal failure who cannot tolerate metformin and SU treatment[31]. Also the repaglinidin's A1C lowering effect is higher than nateglidine.

ALPHA GLUCOSIDASE INHIBITORS

They function by inhibiting carbohydrate absorption by inhibiting the enzyme alpha-glucosidase in the small intestine. The drugs in this group are acarbose, miglitol and voglibose. It is advised to take it before every meal but it can be used at the time of meal with postprandial hyperglycemia (PPH) only because it affects PPH. The most important side effects are swelling, indigestion, diarrhea, reversible increase in liver enzymes and rarely iron deficiency anemia. Contraindications include inflammatory bowel disease, chronic ulceration, malabsorption, partial bowel obstruction and cirrhosis. In addition, treatment should be discontinued in patients with an eGFR of 25 mL/min[32].

INCRETIN BASED MEDICATIONS

Dipeptidyl peptidase-4 inhibitors

Dipeptidyl peptidase-4 inhibitors (DPP-4I) is a member of a large family of enzymes responsible for the destruction of many GIS hormones, neuropeptides, chemokines and cytokines. In response to food-borne carbohydrates, they are secreted from small intestine's K and L cells. They increase pancreatic insulin secretion, slow down gastric emptying, and suppress the increased postprandial glucagon secretion[33]. DPP-4 inhibitors are an attractive treatment option for elderly diabetic patients due to the single daily dose, lack of risk for hypoglycemia and neutral effect on weight. The most important side effects are headache, nasopharyngitis, upper respiratory tract infection and acute pancreatitis. Creatinine clearance should be calculated before treatment in each patient and dose should be reduced if < 50 mL/min. For sitagliptin if Egfr < 30 mL/min for, it should preferably not be used and if the eGFR is between 30-50 mL/min, the dose should be reduced by 50%. If eGFR < 15 mL/min in vildagliptin, saxagliptin, and linagliptin, they should preferably not be used, but for Vildagliptin if eGFR between 30-60 mL/min or patient has dialyzed it can be used without dosage adjustment. For Alogliptin treatment, if the eGFR is 30-60 mL/min, the dose is reduced by 50%, whereas if the eGFR is < 30 mL/min, the dose is reduced by 75%.

In the study comparing glipizide and sitagliptin in elderly diabetic patients, A1C reduction was similar in both groups, but less hypoglycemic event was encountered in the alogliptin-receiving group[34]. In another study conducted by Scirica *et al*[35], the group receiving saxagliptin treatment in elderly diabetic patients was found to have a higher rate of hospitalization with cardiac insufficiency. On the other hand; a systemetic rewiew deduced that incretin-based treatment(agents) does not increase major adverse cardiovascular events[36]. The effects of the DPP-4 group drugs on the A1C lowering are insufficient. Therefore, monotherapy can be used in patients with A1C level close to the target value. They may also be added to metformin, SU and insulin therapy.

Incretinmimetics (glucagon like peptid-1 receptor agonists)

Glucagon like peptid-1 receptor (GLP-1) agonist drugs also target postprandial hyperglycemia and theirhypoglycemia risk is relatively low. However, nausea and weight loss are the most important side effects in vulnerable patients. Other negative features are that they are administered as injections, renal dose reduction and high treatment costs.

Studies have shown that at the end of the 24-wk follow-up period with liraglutide treatment, the decrease in fat mass and lipid profile, as well as the increase in glucose control and insulin sensitivity[37]. In another study about cardiovascular effects of liraglutide treatment, the incidence of non-fatal MI or nonfatal stroke due to cardiovascular causes was found to be lower in patients receiving liraglutide treatment compared to placebo[38].

In another study, the rate of cardiovascular death, non-fatal MI, or non-fatal stroke was found to be significantly lower in type 2 diabetes patients with semaglutide treatment compared to those who received placebo. This result confirms the noninferiority of semaglutidine[39].

Sodium glucose co-transporter 2 inhibitors = glucoretics

Although there isn't any long-term efficacy and safety data available, they may be preferred as treatment options in cases in which sufficient glycemic control is not achieved with dual oral agents such as metformin and SU. Sodium glucose co-transporter 2 inhibitors (SGLT2-I) is secreted in the proximal tubules and reabsorbs about 90% of the glucose load undergoing filtration. The effect of lowering the plasma glucose level and A1C level is limited by the filtered glucose load and osmotic diuresis, and also insulin independent and can be used in any stage of DM2. A1C is reduced by

0.5%-1%. Even though long-term efficacy is unknown, but it has been found that within 2 to 6 mo, it causes about 2 kg of loss, 2-4 mmHg decline in systolic blood pressure, and 1-2 mmHg decline in diastolic KB. It also has the advantages of lowering the level of uric acid and decreased albuminuria and also low risk for hypoglycemia[40]. The most important side effects were increased genital-mycotic infections (11% in females, 4% in males) and an elevated volume gap due to increase in urinary tract infections and diuretic effects[41].

When cardiovascular results of studies with these group drugs were evaluated, there was a significant decrease in the risk of cardiovascular events and cardiovascular caused mortality with Empagliflozin[42]. In the subgroup analysis of the same study, it was found that Empagliflozinhas renoprotectiveeffects and reduced the risk for development of renal complications[43]. The study of the concurrent use of DPP-4I and SGLT2-I treatment showed that monotherapy was more effective in patients treated with metformin, when a gliptin was added to gliflozin, it was determined that it had the effect of more glucose lowering than when a gliflozin was added to gliptin. The opinion at the end of the meta-analysis is that SGLT2I and DPP-4I can be used as an initial combination or as a gradual approach and combining two pharmacological options is safe and does not induce hypoglycemia[44]. Furthermore, when the sum of the evidence for the use of dapagliflozin is assessed, it has not been shown that there is a causal relationship between dapagliflozin and bladder cancer, which was previously proposed[45].

Should be used more carefully in elderly because for causing osmotic diuresis resulting dehydration, causing an increase in the frequency of genital and urinary system infections, weight loss, dose adjustment necessity in renal failure and lack of enough data on microvascular and cardiovascular outcomes.

INSULIN THERAPY

In the elderly with poor glycemic control, HbA1c level > 9% (74.9 mmol/mol), FPG level > 250 mg/dL (13.9 mmol/L), randomized glucose value > 300 mg/dL or patients with ketonuria, insulin should be selected as initial therapy. When starting insulin therapy in elderly patients, it is important to have general health status, ability to make insulin, to measure blood sugar, to understand hypoglycemia and capacity to treat it. In the study of geriatric patients using basal insulin and OAD, treatment-related satisfaction surveys and post-12-wk follow-up in the insulin treatment group showed significant improvement in the geriatric depression scale (SOURCE). In another study, geriatric patients were divided into OAD treatment with basal insulin addition and elevated OAD dosage group, and 24-mo follow-up revealed a lower frequency of hypoglycemia in the basal insulin group (SOURCE). In a randomized controlled trial in long-term care patients, basal insulin was added to a group and OAD added to another group and glycemic control and development of hypoglycemia were evaluated and no significant difference was detected. When the ready mixed insulins are evaluated, they are more effective for the control of postprandial glycaemia, but they are more useful for the patients who live in the nursing home, who eat regular meals.

New oral glucose-lowering agents are less likely to have all-cause mortality, CVD, and severe hypoglycemia when compared to insulin. Dapagliflozin has both decreased mortality due to all causes and reduced CVD risk, while DPP-4i has been found to be weaker in decrease of all cases due to mortality[46].

Elderly patients have their own nutritional needs.Along with the increased age, the taste and odor sensations diminishes, as well as changes in the threshold of thirst. For this reason, the balance between pre-meal insulin and oral food intake should be well established in elderly patients. Insulin dose reduction should be done according to the amount of carbohydrates taken at meals, for example if half of the meal is consumed, insulin will be reduced by 50%, insulin will not be administered or 25% can be administered to patients who consume less than that or may skip meals due to a medical intervention. In addition, patients with enteral or parenteral nutrition should be monitored for glucose at 4-6 h intervals to avoid hypo-hyperglycaemia[47].

CONCLUSION

When starting OAD or insulin therapy in the elderly, treatment regimens containing as simple and few drugs as possible should be administered, and drug therapy should not be initiated unless it is necessary and if necessary must start with low dose and dose increase should be done slowly. All patients should be evaluated with liver and kidney function tests before onset of treatment.

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ORIGINAL ARTICLE

Observational Study

Statin use and cognitive function in middle-aged adults with type 1 diabetes

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Abstract

AIM

To test associations between statin use and cognitive impairment in adults with childhood-onset type 1 diabetes (T1D).

METHODS

In 2010-13, *n* = 108 middle-aged participants from ongoing observational Pittsburgh Epidemiology of Diabetes Complications Study underwent neurocognitive assessment (mean age and T1D duration of 49 and 41 years, respectively). All were diagnosed with childhood-onset (*i.e.*, prior to age 18) T1D between 1950 and 1980 and were seen within one year of diagnosis at Children's Hospital of Pittsburgh. Self-reported statin use (yes/no and if yes, name of statin) was collected biennially from parent study baseline (1986-1988) to time of neurocognitive testing. Logistic regression models tested associations between statin use groups and cognitive impairment (defined as having two or more cognitive test scores 1.5SD or worse than published norms) while linear regression models tested associations between statin use groups and cognitive domain z-scores (domains: Verbal IQ, memory, executive function, psychomotor speed, and visuo-

construction). All models controlled for education and age. To address confounding by indication, models were repeated using a propensity score for statin use.

RESULTS

Of the 108 participants, 51 reported never using statins. Median duration of statin use among the 57 ever users was 6 years. These 57 ever statin users were split to create two groups (⩽ or > median years of statin use): 1-6 years (n = 25), and 7-12 years (n = 32). Compared with never users, using statins 1-6 years tripled the odds of cognitive impairment (OR = 3.16; 95%CI: 0.93-10.72; P = 0.06) and using statins 7-12 years almost quintupled the odds of cognitive impairment (OR = 4.84; 95%CI: 1.63-14.44; P = 0.005). Compared with never users, using statins 1-6 or 7-12 years was related to worse performance in the memory domain (β = -0.52; P = 0.003, and -0.39; P = 0.014, respectively). Adjusting for coronary artery disease, low density lipoprotein cholesterol, and *Apo E4* status did not substantially alter results, and none of these covariates were significantly related to cognitive outcomes (all P > 0.05). Propensity score analyses support that associations between poor cognitive outcomes and statin use were not due merely to confounding by indication.

CONCLUSION

Statin use was associated with cognitive impairment, particularly affecting memory, in these middle-aged adults with childhood-onset T1D, whom at this age, should not yet manifest age-related memory deficits.

Key words: Type 1 diabetes; Cognitive impairment; Memory; Statin use; Cohort study

Core tip: Animal and cell culture studies show that statins can damage cerebral gray and white matter, thereby affecting cognitive function. Findings from human studies remain controversial; early observational studies reported that statin use negatively affected cognition, especially memory, while more recent studies have not replicated these findings. Even though statins are widely prescribed for people with type 1 diabetes (T1D), only one study to date has examined whether statin use is related to cognitive impairment in this patient population. We propose that deleterious effects statins may exert on cognition may be more pronounced in people with T1D, as these individuals are already at an increased risk of cognitive impairment due to long-term exposure to metabolic dysregulation.

Nunley KA, Orchard TJ, Ryan CM, Miller R, Costacou T, Rosano C. Statin use and cognitive function in middle-aged adults with type 1 diabetes. *World J Diabetes* 2017; 8(6): 286-296 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i6/286.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i6.286

INTRODUCTION

Whether statins negatively affect cognitive function remains under dispute. Goldstein and Mascitelli[1] (2014) propose that statins may negatively affect the brain and cognitive health, potentially *via* impaired myelination. Additionally, cell culture and animal studies show that statins exert neurotoxic effects[2,3]. Four recent meta-analyses/reviews, however, found no significant relationship between statin use and cognitive impairment[4-7]. While these reviews do acknowledge that statins may negatively impact cognitive function in "vulnerable" populations, they provide no insight as to who may be "vulnerable". We raise the possibility that adults living with type 1 diabetes (T1D) since childhood may fit this "vulnerable" category, for at least two reasons.

First, a growing body of literature recognizes the deleterious effects of T1D on brain structure, with smaller total brain volume reported among those with than those without T1D[8-10]. Perhaps negative effects of statins of brain function are more pronounced in those with overall smaller brain volume. In other words, those with greater cerebral gray and white matter volumes may be more able to compensate for insults to cerebral gray or white matter related to statin use.

Second, to minimize cardiovascular events, the American Diabetes Association recommends moderate to high intensity statin treatment for diabetic patients at any age who also have atherosclerotic cardiovascular disease, or its risk factors (*e.g*., hypertension, dyslipidemia, overweight/obese), and for all diabetic patients aged 40 years and older, regardless of cardiovascular risk[11]. This means that many T1D patients begin using statins in early adulthood, often before age 30, whereas statin use is relatively uncommon among otherwise "healthy" adults under age 45. While youth with neurofibromatosis 1 or familial hypercholesterolemia also use statins at an early age, the long-term effects of statin use on cognitive function in these patients also remains unclear[12]. In fact, a recent randomized controlled trial recommends against using simvastatin to enhance cognitive function in children with neurofibromatosis 1[13]. Age at initial statin exposure is an important consideration because the brain's white matter continues to undergo myelination well into the 4th decade of life[14,15]. If statins do compromise myelin integrity, then statin use may differentially impact the brain depending on the age at which statin use begins. Additionally, long-term statin use may also reduce the number of glial progenitor cells available for future recruitment as these patients age[16]. Thus, exposure to statins prior to age 40 years, in combination with the metabolic dysregulation that accompanies T1D, may noticeably disrupt brain myelination or myelin integrity, whereas little to no discernable disruption of brain myelin/myelination occurs when delaying exposure to statins until after age 50, and/or in the absence of T1D.

Despite this unique statin use prolife of T1D patients, we found only one study to examine statins and cogni-

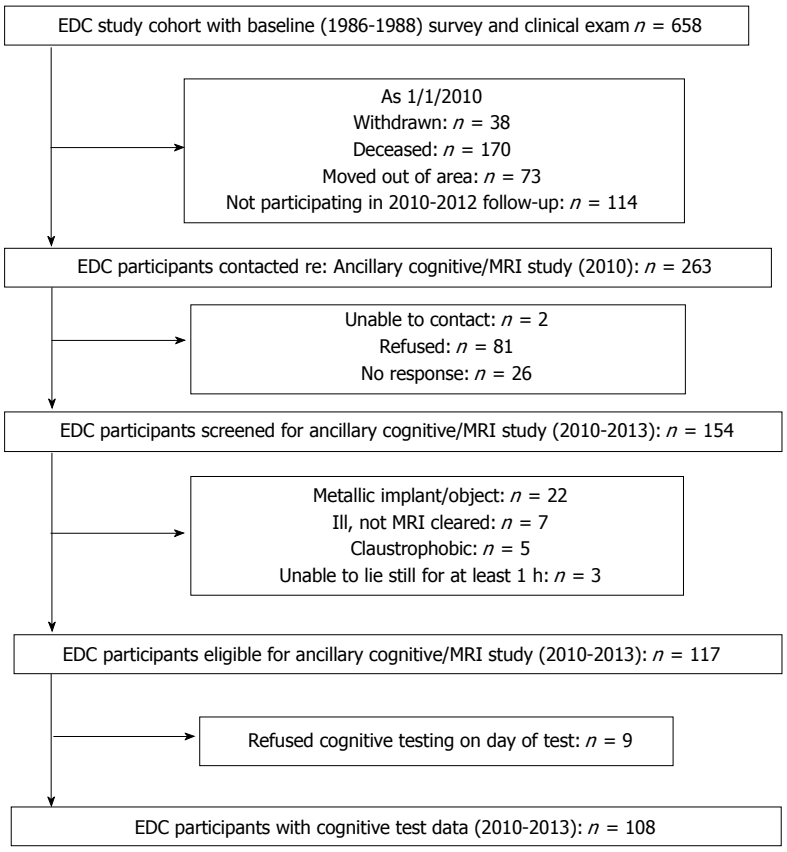

Figure 1 Recruitment of participants with type 1 diabetes from the parent Pittsburgh Epidemiology of Diabetes Complications Study into the ancillary neurocognitive study. EDC: Epidemiology of Diabetes Complications.

tive function in adults with T1D[17]. This small study found no association between statin use and cognitive impairment. However, only 11 out of 55 cases used statins, and duration of statin use was not examined.

We recently documented a higher-than-expected prevalence of cognitive impairment in the middle-aged T1D cohort currently being reported[18], but did not examine statin use as a risk factor for cognitive impairment. This cross-sectional study was therefore conducted to determine whether statin use was associated with cognitive impairment in middle-aged adults with childhood-onset T1D.

MATERIALS AND METHODS

Participants

This study sample was recruited from the Pittsburgh Epidemiology of Diabetes Complications (EDC) Study, an on-going, prospective observational study of individuals diagnosed with childhood-onset (< age 17 years) T1D between 1950 and 1980, and drawn from the Children's Hospital of Pittsburgh diabetes registry. During 2010-2013, an MRI eligible subset (108 out of 261 living in the Pittsburgh area, Figure 1) participated in an ancillary neuroimaging and neurocognitive study.

Cognitive assessment

Details and results comparing cognitive impairment between this T1D cohort and 138 similarly-aged adults without T1D have been previously published (Nunley *et al*[18], 2015). In brief, both cohorts underwent a neurocognitive test battery to assess verbal IQ (North American Adult Reading Test); memory [Rey Auditory Verbal Learning Test - immediate, delay and interference trials, Rey-Osterrieth Complex Figure Delay Task (ROCF-Delay), and Four Word Short Term Memory 5-, 15- and 3-s lists]; executive function [Verbal Fluency F-A-S (FAS), Stroop Color-Word (Stroop-CW), Trails Making B (TMTB), Ratio TMTB: TMTA, Letter-Number Sequence]; psychomotor speed [Digit Symbol Substitution Test (DSST), Grooved Pegboard (GP), Trail Making Test A (TMTA)]; semantic fluency [Verbal Fluency Animals (Animals)]; and visuo-construction [Rey-Osterrieth Complex Figure Copy Task (ROCF-copy)]. In addition to calculating standardized scores for each domain, raw scores on each task were compared to published, demographically-appropriate means[19-21]. T1D cases performed significantly worse than non-T1D controls on seven tasks: FAS, TMTB, DSST, GP, Stroop-CW, Animals, and ROCF-copy. Any participant scoring 1.5 SD or worse than demographically-appropriate published norms on two or more of these seven tasks met the study definition of cognitive impairment[18]; this classification of cognitive impairment (scores worse than 1.5SD) has been previously validated[22].

Statin use

Participants self-reported all medication use biennially, from parent study baseline (1986-1988) through time of cognitive testing (2010-2013). Statin type was determined using Anatomical Therapeutic Chemical Classification System coding (ATC code): ATC codes

C10AA01, 02, and 05, or combination drugs using simvastatin, atorvastatin, or lovastatin, were classified as lipophilic, while codes C10AA03, 04 and 07, or combination drugs using pravastatin or rosuvastatin, were classified as hydrophilic.

Depression/depressive symptoms

Participants completed the Beck Depression Inventory at time of cognitive testing; scores $\geqslant$ 10 were categorized as positive for depressive symptoms[23].

Risk factors

Serum total and HDL cholesterol levels were assessed, using standardized methods, at each clinic visit from parent study baseline (1986-1988) to time of cognitive testing (2010-2013); low density lipoprotein cholesterol (LDLc) was calculated using the Friedwald equation. Details on methods of assessing lifestyle/medical factors (*e.g.*, blood pressure, diabetes complications, inflammatory markers) have been described elsewhere (for details, see Pambianco *et al*[24], 2006).

Brain imaging markers

Severity of cerebral white matter hyperintensities (Fazekas rating 2-3 *vs* Fazekas 1) served as markers of cerebral small vessel disease; for details of image acquisition and rating of white matter hyperintensities, see Nunley *et al*[25], 2015. Left hippocampal volume, as a percentage of total intracranial volume, was chosen for these analyses as hippocampal volume is positively related to memory performance; for details of gray matter imaging and segmentation, see Hughes *et al*[26], 2013.

Statistical analysis

Participants with neurocognitive data (n = 108) were compared with the remaining 154 participants from the parent study who were MRI ineligible, unable to schedule, or not interested in the neurocognitive study. Data from the parent study's 2004-2006 exam were used to compare participant characteristics, including statin use (yes/no). This time point was selected because it was the most recent physical exam for participants who did not participate in neurocognitive study (*i.e.*, only the subgroup participating in the neurocognitive exam underwent a physical exam in 2010-2013, while all participants were offered a physical exam in 2004-2006).

Participants with neurocognitive data were categorized into three groups, based on the distribution of duration of statin use: Never (0 years); 1-6 years; and 7-12 years. This created two groups of ever statin users, split by the median years of statin use. Lipophilic statin use was also determined for all statin users. Characteristics of the three groups were compared using ANCOVA, Fisher exact test, and Jonckheere-Terpstra test as appropriate. *T* tests, Fisher exact, and Wilcoxon Rank-Sum tests compared select factors between participants by cognitive impairment status, as appropriate. Age- and education-adjusted *P* values were obtained from ordinal logistic regression models.

Logistic and linear regression models tested the association between statin use (covariate of interest, with never users as the referent group) and cognitive impairment or cognitive domain z-scores (outcomes). All models controlled for age and education, as we previously demonstrated that education was highly associated with cognitive impairment in this cohort[18]. Each candidate explanatory factor (*i.e.*, related to statin use with a $P \leqslant 0.10$) was entered individually into the model(s); this approach was necessary due to the high degree of multicollinearity between most factors. Underlying brain pathology markers (white matter hyperintensity severity, left hippocampal volume) were forced separately into the models. To arrive at the most parsimonious models, only factors associated with the outcome at $P \leqslant 0.05$ were retained and presented in the tables, controlling for age and education.

Lastly, to account for possible confounding by indication and given the limited sample size of the study, we calculated a propensity score covariate to control for the group difference in statin use. The propensity score was generated based on multinomial logistic regression with the following covariates: Diastolic blood pressure, LDLc, body mass index, smoking history, and history of high blood pressure/using anti-hypertensive medications. Relationships between duration of statin use with cognitive impairment and memory domain z-score were then assessed by logistic regression and linear regression, respectively, while adjusting for the propensity score, age and education.

All participants provided informed consent prior to all study procedures. The University of Pittsburgh IRB approved the study. SAS 9.3 (Cary, NC) was used for data analyses. A biostatistician from University of Pittsburgh Medical Center, Dr. Yuefang Chang, was consulted and contributed to the statistical analyses for this study.

RESULTS

Statin use, duration of statin use, study-average LDL cholesterol, history of high blood pressure, and glycemic control did not differ significantly between those who participated in the neurocognitive study and those unable, ineligible, or refusing participation in the ancillary neurocognitive study (Table 1, all $P > 0.10$). Those who agreed to participate had marginally shorter diabetes duration and were generally healthier (*e.g.*, lower prevalence rates of retinopathy, neuropathy, microalbuminuria, coronary artery disease) than those who did not participate (Table 1, all $P < 0.02$).

Of the 108 with cognitive data, a single participant first reported statin use in 1990-1992; a second participant reported statin use in 1996-1998. Statin use increased at each successive biennial exam, with a total

Table 1 Adults with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study, by participation status in the ancillary neurocognitive study

	Non-participant (*n* = 154)	Participant (*n* = 108)	*P* value
Demographic and lifestyle factors, data are *n* (%), mean ± SD, or median (IQR)			
Age (yr)	51.17 ± 7.74	49.52 ± 7.04	0.08
Female	86/136 (63%)	55 (51%)	0.07
Years of education	14 ± 2	15 ± 3	0.05
Ever smoking 100 + cigarettes[1]	57/136 (42%)	41 (38%)	0.60
ApoE4 (24, 34, 44)	34/151 (23%)	34 (32%)	0.12
BMI (kg/m^2)	27.52 ± 4.88	26.74 ± 4.26	0.20
Depressive symptoms[2]	45/128 (35%)	23/100 (23%)	0.06
Physical activity (Kcal)[3]	729 (308-1663)	1009 (448-1966)	0.05
Type 1 diabetes-related factors			
T1D duration (yr)	37.14 ± 7.20	35.50 ± 6.32	0.07
Age at diagnosis (yr)	8.62 ± 4.10	8.28 ± 4.11	0.51
HbA1c (%)	7.69 ± 1.69	7.85 ± 1.85	0.51
A1c months (AU)	1036.38 ± 481.55	966.82 ± 382.02	0.21
Insulin sensitivity (eGDR, mg/kg per minute)	7.65 ± 2.11	7.68 ± 2.47	0.94
eGFR (mL/min per 1.73 m^2)	77.49 ± 24.41	83.31 ± 24.06	0.09
Proliferative retinopathy	85/131 (65%)	51/107 (48%)	0.009
Microalbuminuria	98/133 (74%)	54/92 (59%)	0.02
Coronary artery disease	48 (31%)	18 (17%)	0.009
Cardiac autonomic neuropathy	89/125 (71%)	48/97 (49%)	0.001
Distal symmetric polyneuropathy	86/128 (67%)	52/100 (52%)	0.02
Cardio-metabolic factors			
Systolic blood pressure (mmHg)	116 ± 17	114 ± 16	0.28
Diastolic blood pressure (mmHg)	65 ± 10	66 ± 11	0.42
History of high blood pressure[4]	71 (46%)	39 (36%)	0.13
Total cholesterol (mg/dL)	174.07 ± 34.92	174.79 ± 35.85	0.88
LDL cholesterol (mg/dL)	98.15 ± 28.44	98.48 ± 33.72	0.94
HDL cholesterol (mg/dL)	59.89 ± 16.31	60.63 ± 16.68	0.74
Serum creatinine (mg/dL)	1.12 ± 0.67	1.07 ± 0.61	0.57
Ever used statins[1]	97 (63%)	57 (53%)	0.13
Years of statin use[1]	3 (0-6)	2 (0-8)	0.44
Study average LDLc (mg/dL)[1]	109.95 ± 23.28	107.65 ± 25.96	0.45
Inflammatory markers			
WBC × 10^3/mm^2	6.2 (4.9-7.8)	6.1 (5.2-6.9)	0.30
Adiponectin (μg/mL)	21.1 (15.2-31.0)	22.2 (15.2-30.1)	0.83
IL-6 (ng/mL)	1.4 (0.8-2.3)	1.3 (0.8-1.8)	0.42
TNFα (pg/mL)	1.3 (1.0-1.9)	1.3 (1.0-1.8)	0.92
C-reactive protein (mg/L)	1.7 (0.9-3.3)	1.1 (0.6-2.5)	0.03

[1]Assessed repeatedly from 1986-88 (baseline) through 2004-2006; [2]Beck Depression Inventory score ⩾ 10; [3]Estimated self-reported weekly activity per modified Paffenbarger questionnaire; [4]Blood pressure > 140/80 at any physical exam as part of the parent study and/or any self-reported use of anti-hypertensive medication (1986-2006). Factors assessed in 2004-2006 unless otherwise specified. T1D: Type 1 diabetes; LDLc: Low density lipoprotein cholesterol; BMI: Body mass index; eGDR: Estimated glucose disposal rate; WBC: White blood cell count; IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha.

of 57/108 classified as "ever" statin users (Figure 2). Of ever statin users, 51/57 (89%) used only lipophilic statins; the small number using hydrophilic statins did not allow for meaningful comparisons by statin type.

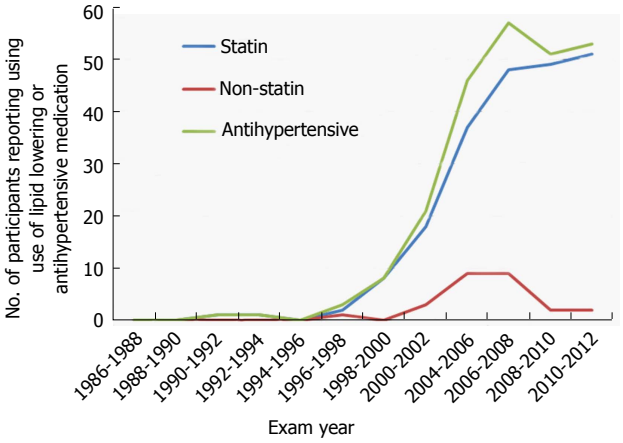

Figure 2 Numbers of participants with type 1 diabetes in the ancillary neurocognitive study (*n* = 108) who reported using lipid lowering and antihypertensive medications from parent study baseline (1986-1988) through time of cognitive assessment (2010-2012).

Of the 51 "never" statin users, six individuals reported using a non-statin alternative (*e.g.*, nicotinic acid) to control their cholesterol.

The three statin use groups did not significantly differ (Table 2, all $P > 0.05$) in male:female ratio, education, *ApoE4* allele status, estimated weekly physical activity, presence of depressive symptoms, age at T1D diagnosis, serum glucose at time of cognitive testing, prevalent cardiac autonomic neuropathy, distal symmetric polyneuropathy, history of stroke, systolic or diastolic blood pressure, average ankle:brachial index > 1.3 or non-compressible[27], or concentrations of white blood cell count, adiponectin, or IL-6. Longer duration of statin use was significantly and positively associated with age, BMI, T1D duration, and study-average LDLc concentration, and was significantly and negatively associated with insulin sensitivity (per estimated glucose disposal rate), and kidney function (estimated glomerular filtration rate). Increasing duration of statin use was associated with a lower prevalence of smoking and with a higher prevalence of coronary artery disease and proliferative retinopathy, of having a 14-year average A1c > 7.5% (> 58 mmol/mol), and of having a history of high blood pressure or using anti-hypertensive medication (Table 2, all $P < 0.05$).

A total of 30/108 (28%) participants met the study definition of cognitive impairment[18] and the percentage of participants with cognitive impairment increased with increasing duration of statin use: 14% of never users, 32% of 1-6 years of statin use, and 47% of 7-12 years of statin use (Table 2, $P = 0.003$). Longer duration of statin use was significantly related to worse performance on memory (Table 2, $P = 0.004$) and psychomotor speed (Table 2, $P = 0.012$), but no other domains (Table 2, all $P > 0.05$).

Cognitively impaired participants were significantly more likely to have coronary artery disease, a history of ever using statins, and for a longer duration, than

Table 2 Comparison of middle-aged adults with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study by duration of statin use

	Never used (*n* = 51)	1-6 yr (*n* = 25)	7-12 yr (*n* = 32)	*P* value[1]
Demographic and lifestyle factors, data are *n* (%), mean ± SD, or median (IQR)				
Age at cognitive testing (yr)	47.5 ± 7.3	51.8 ± 6.1	51.0 ± 6.7	0.02
Female	27 (53%)	16 (64%)	12 (38%)	0.10
Years of education	15 ± 2	16 ± 3	14 ± 3	0.52
Ever smoking 100+ cigarettes[5]	22 (43%)	11 (44%)	8 (25%)	0.05
Apo E4 (24, 34, 44)	16 (31%)	7 (28%)	11 (34%)	0.66
BMI (kg/m^2)	26.0 ± 4.3	27.6 ± 5.1	29.8 ± 4.7	0.002
Cognitive function				
Cognitively impaired	7 (14%)	8 (32%)	15 (47%)	0.003
Estimated verbal IQ	108.6 ± 8.2	107.7 ± 10.0	106.5 ± 6.9	0.24
Memory domain z-score	0.24 ± 0.75	-0.23 ± 0.64	-0.25 ± 0.78	0.004
Executive function z-score	0.18 ± 0.56	-0.10 ± 0.82	-0.30 ± 0.79	0.06
Psychomotor speed z-score	0.29 ± 0.66	-0.33 ± 1.10	-0.28 ± 0.89	0.01
Visuo-construction z-score	0.21 ± 0.64	-0.16 ± 0.82	-0.21 ± 1.45	0.13
Type 1 diabetes-related factors				
Diabetes duration (yr)	39.6 ± 5.8	43.4 ± 6.9	42.1 ± 6.5	0.03
Serum glucose (mg/dL)	188.6 ± 90.5	151.1 ± 73.6	173.0 ± 81.8	0.56
A1c > 7.5%, 14-yr average	27 (53%)	17 (68%)	25 (78%)	0.02
Glucose disposal rate (mg/kg per minutr)[2]	8.1 ± 2.0	7.5 ± 1.8	5.8 ± 2.9	< 0.001
Proliferative retinopathy[2]	17 (33%)	14 (58%)	20 (63%)	0.03
eGFR (mL/min per 1.73 m^2)[2,4]	91.3 ± 21.1	79.7 ± 20.1	74.7 ± 27.5	0.02
Coronary artery disease[2]	5 (10%)	3 (12%)	10 (31%)	0.02
Cardiac autonomic neuropathy[2]	21 (47%)	14 (58%)	13 (46%)	0.36
Distal symmetric polyneuropathy[2]	22 (49%)	13 (57%)	17 (53%)	0.61
Cardio-metabolic factors				
History of stroke[5]	1 (2%)	2 (8%)	2 (6%)	0.99
Systolic blood pressure (mmHg)	117.6 ± 12.0	119.6 ± 15.5	123.2 ± 19.3	0.44
Diastolic blood pressure (mmHg)	65.0 ± 9.5	64.6 ± 9.1	67.5 ± 10.6	0.18
History of high blood pressure[3]	13 (25%)	10 (40%)	16 (50%)	0.04
Study average LDLc (mg/dL)[5]	100.3 ± 25.6	112.2 ± 24.9	115.9 ± 24.7	0.02
Inflammatory markers				
[2]WBC × 10^3/mm^2	5.9 (5.0-6.7)	6.2 (5.2-6.9)	6.2 (5.2-7.1)	0.29
Adiponectin (μg/mL)[2]	22.0 (15.7-30.7)	21.8 (14.2-31.4)	22.3 (15.2-28.3)	0.75
IL-6 (ng/mL)[2]	1.4 (0.7-1.9)	1.2 (0.8-1.7)	1.2 (1.0-1.6)	0.28
TNFα (pg/mL)[2]	1.3 (1.0-2.3)	1.2 (1.0-1.8)	1.3 (1.0-1.6)	0.07
C-reactive protein (mg/L)[2]	0.9 (0.6-2.3)	0.9 (0.2-1.6)	1.9 (0.6-4.1)	0.08

[1]*P* values are adjusted for age and education; [2]Assessed in 2004-2006; [3]Defined as any EDC assessed SBP > 140 mmHg or DBP > 90, or ever self-reported use of anti-hypertensive medication from 1986-1988 through 2010-2013; [4]Estimated per the Chronic Kidney Disease - Epidemiology (CKD-EPI) formula; [5]Assessed from EDC baseline (1986-1988) through time of cognitive testing (2010-2013). Factors assessed at time of cognitive testing (2010-2013) unless otherwise specified. T1D: Type 1 diabetes; LDLc: Low density lipoprotein cholesterol; BMI: Body mass index; eGDR: Estimated glucose disposal rate; WBC: White blood cell count; IL-6: Interleukin-6; TNFα: Tumor necrosis factor alpha.

cognitively normal participants, independent of education (Table 3 all $P < 0.05$). While not statistically significant, cognitively impaired participants were more likely to have a higher study-average LDLc as compared with cognitively normal participants (Table 3, $P = 0.063$). Associations between cognitive impairment and history of high blood pressure/using anti-hypertensive medication and brain imaging data were not statistically significant (Table 3, all $P > 0.10$) (for details regarding relationships between other risk factors and cognitive impairment in this cohort, see references[18,28]).

In logistic regression models with cognitive impairment as the outcome, using statins for 1-6 years, as compared with never using statins, more than tripled the odds of cognitive impairment, but was only marginally significant after controlling for age and education (Table 4, Model 1). Compared with never using statins, statin use of 7-12 years was related to almost five-fold higher odds of cognitive impairment, independent of age or education (Table 4, Model 1). Controlling for long-term LDLc, coronary artery disease, or *Apo E4* allele status did not substantially alter the relationship between duration of statin use and cognitive impairment. Furthermore, LDLc, coronary artery disease, and *Apo E4* allele status were not significantly related to cognitive impairment (Table 4, Models 2-5). Results were overall unchanged when adjusting for white matter hyperintensities or left hippocampal volume (data not shown).

In linear regression models with memory domain z-score as the outcome, using statins for 1-6 years was related to half a SD decrease in memory domain score (Table 5, Model 1) as compared with never using statins. Using statins for 7-12 years was related to almost half a SD decrease in memory domain score (Table 5, Model 1) as compared with never using statins. Controlling for LDLc, coronary artery disease, or *Apo E4*

Table 3 Select characteristics[1] of middle-aged adults with childhood-onset type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study, by cognitive impairment status

	Cognitively normal (*n* = 78)	Cognitively impaired (*n* = 30)	*P* value
Data are *n* (%), mean ± SD, or median (IQR)			
Coronary artery disease[2]	9 (12%)	9 (30%)	0.02
Cardio-metabolic risk factors			
Ever using statins (1986-2013)[3]	34 (44%)	23 (77%)	0.003
Duration of statin use (statin years)[3]	0 (0-6)	7 (2-8)	0.002
If statin use, only used lipophilic statin[3]	30 (88%)	21 (91%)	0.99
Study average LDLc (mg/dL)[3]	104.5 ± 25.8	115.9 ± 24.8	0.06
History of high blood pressure[4]	26 (33%)	13 (43%)	0.24
Brain imaging			
Severe White Matter Hyperintensities[5]	17 (26%)	11 (46%)	0.09
Left hippocampal volume[6]	0.31 ± 0.03	0.31 ± 0.03	0.31

Reported *P* value is adjusted for education. [1]Relationships between other factors and cognitive impairment in this type 1 diabetes cohort have been previously described and published elsewhere (for details, see Nunley *et al*[18], 2015); [2]Assessed in 2004-2006; [3]Assessed since EDC baseline (1986-1988) through time of cognitive testing (2010-2013); [4]Defined as any EDC assessed SBP > 140 mmHg or DBP > 90, or ever self-reported use of anti-hypertensive medication from 1986-1988 through 2010-2013; [5]Fazekas rating 2-3 *vs* Fazekas rating 1; data on *n* = 89 (for details, see Nunley *et al*[28], 2015); [6]Hippocampal volume as a percentage of total intracranial volume, data on *n* = 88 (for details, see Hughes *et al*[26], 2013). Measures assessed 2010-2013 unless otherwise noted. LDLc: Low density lipoprotein cholesterol.

Table 4 Results of logistic regression models assessing the association between duration of statin use and cognitive impairment in middle-aged adults with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study

	Variables in Model	Cognitive impairment OR (95%CI) *P* value
Model 1	Never used statins	Referent group
	1-6 yr statins	3.16 (0.93-10.72), *P* = 0.064
	7-12 yr statins	4.84 (1.63-14.44), *P* = 0.005
Model 2	Never used statins	Referent group
	1-6 yr statins	2.86 (0.83-9.86), *P* = 0.095
	7-12 yr statins	4.26 (1.40-13.00), *P* = 0.011
	Average LDLc	1.01 (0.99-1.03), *P* = 0.24
Model 3	Never used statins	Referent group
	1-6 yr statins	3.29 (0.95-11.40), *P* = 0.061
	7-12 yr statins	4.13 (1.35-12.60), *P* = 0.013
	CAD	2.88 (0.88-9.44), *P* = 0.081
Model 4	Never used statins	Referent group
	1-6 yr statins	3.14 (0.93-10.64), *P* = 0.066
	7-12 yr statins	4.95 (1.65-14.82), *P* = 0.004
	Apo E4 allele	0.73 (0.26-2.02), *P* = 0.55
Model 5	Never used statins	Referent group
	1-6 yr statins	2.90 (0.82-10.29), *P* = 0.099
	7-12 yr statins	3.69 (1.17-11.68), *P* = 0.026
	Average LDLc	1.01 (0.99-1.03), *P* = 0.24
	CAD	2.72 (0.81-9.13), *P* = 0.11
	Apo E4 allele	0.75 (0.26-2.15), *P* = 0.59

Statin use groups: Never used *n* = 51; 1-6 years *n* = 25; 7+ years *n* = 32. Binary outcome: Cognitive impairment present/absent. Model 1: Statin use groups, controlling for age and education; Model 2: Model 1, further controlling for average long-term LDLc (1986-1988 through 2010-2013); Model 3: Model 1, further controlling for prevalent coronary artery disease (CAD); Model 4: Model 1, further controlling for Apo E4 allele status (24, 34, or 44); Model 5: Model 1, further controlling for LDLc, CAD, and Apo E4 allele. LDLc: Low density lipoprotein cholesterol.

allele did not substantially alter the relationship between duration of statin use and lower memory domain score, and none of these factors were significantly related to memory domain score (Table 5, Models 2-5). Results were independent of brain imaging markers (data not shown).

Using propensity score analyses, those using statins for 1-6 years or for 7-12 years were three times more likely to have cognitive impairment as compared with never statin users; the association was borderline significant for those using statins 1-6 years (OR = 3.48, 95%CI: 0.97-12.51; *P* = 0.056) while the association was statistically significant for those using statins 7-12 years (OR = 3.62, 95%CI: 1.05-12.49; *P* = 0.042). Compared with never statin users, using statins for 1-6 years was statistically significantly related to worse memory z-score (Beta: -0.47, SE = 18, *P* = 0.012). While memory domain z-scores were lower for those using statins for 7-12 years than for never users, the difference did not reach statistical significance (Beta: -0.29, SE = 0.18; *P* = 0.12).

DISCUSSION

This study analyzed correlations between statin use and cognitive impairment in a sub-group of participants with T1D from the on-going, observational Pittsburgh Epidemiology of Diabetes Complications Study. These now middle-aged adults were diagnosed with T1D prior to age 18 years, and have reported medication use biennially since the parent study baseline in 1986. Among the 108 participants with a cognitive assessment in 2010-2013, using statins more than tripled the odds of having cognitive impairment discernible by middle age. As duration of statin use increased (never, 1-6 years, 7-12 years), an increasing percentage of participants met the study definition of cognitive impairment (14%, 32% and 47%, respectively), independent of age or education. Depressive symptoms were not associated with statin use, and we have previously shown depressive symptoms were not related to cognitive impairment in this cohort[28]. Results were robust to adjustment for prevalent coronary artery disease, *Apo E4* status, and long-term average LDL cholesterol concentration.

Our results contradict those reported by the only other study we know of to examine relationships between statin use and cognitive function in T1D cohort[17]. This could be due to several factors, including the small number of participants in the prior study who used statins (11 out of 55), the younger age of their participants (mean age 39 years), or that their study population included T1D cases diagnosed in adulthood (diabetes duration ranged from 6-35 years)[17], whereas our cases were all diagnosed in childhood. Furthermore,

Table 5 Results of linear regression models assessing the association between duration of statin use and memory domain function in middle-aged adults with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study

	Variables in Model	Memory domain standardized β, *P* value
Model 1	Never used statins	Referent group
	1-6 yr statins	-0.284, *P* = 0.003
	7-12 yr statins	-0.232, *P* = 0.01
Model 2	Never used statins	Referent group
	1-6 yr statins	-0.267, *P* = 0.006
	7-12 yr statins	-0.209, *P* = 0.031
	Average LDLc	-0.084, *P* = 0.34
Model 3	Never used statins	Referent group
	1-6 yr statins	-0.267, *P* = 0.006
	7-12 yr statins	-0.213, *P* = 0.032
	CAD	0.02, *P* = 0.86
Model 4	Never used statins	Referent group
	1-6 yr statins	-0.284, *P* = 0.003
	7-12 yr statins	-0.231, *P* = 0.014
	Apo E4 allele	-0.01, *P* = 0.92
Model 5	Never used statins	Referent group
	1-6 yr statins	-0.267, *P* = 0.007
	7-12 yr statins	-0.213, *P* = 0.034
	Average LDLc	-0.084, *P* = 0.35
	CAD	0.02, *P* = 0.86
	Apo E4 allele	-0.001, *P* = 0.99

Statin use groups: Never used n = 51; 1-6 years n = 25; 7-12 years n = 32. Outcome: Standardized score of seven tasks assessing memory domain (z-score, in SD units). Model 1: Statin use groups, controlling for age and education; Model 2: Model 1, further controlling for average long-term LDLc (1986-88 through 2010-2013); Model 3: Model 1, further controlling for prevalent coronary artery disease (CAD) as of 2004-2006; Model 4: Model 1, further controlling for *Apo E4* allele status (24, 34, or 44); Model 5: Model 1, further controlling for LDLc, CAD, and *Apo E4* allele. LDLc: Low density lipoprotein cholesterol.

the prior study did not provide information on duration of statin use in their T1D participants.

That statin use in our cohort was associated with poor performance of memory tasks is of particular interest for three reasons. First, memory problems are the most commonly reported cognitive complaint among statin users[29-32]. Second, with a mean age of 49 years, our T1D participants should not yet exhibit memory deficits commonly observed in adults ages 65 and older[33]. And third, our findings contradict prior reports that memory appears to be preserved in adult T1D populations[34-36]. Considering these three points, we believe additional studies are warranted to investigate the cognitive effects of statin use, along with other potential risk factors related to cognitive impairment and poor memory, in adults with childhood-onset T1D. Such studies should employ a longitudinal design, assessing cognitive performance repeatedly, with at least one done prior to initiating statin use, and with detailed ascertainments of statin use (*e.g.*, type, dose, age at initiation) over time. We believe this should be a public health priority given that the improved life expectancy of people with T1D[37] will lead to a rapidly-growing population of aging adults with T1D who are at risk of cognitive impairment, with high personal and societal costs.

While confounding by indication cannot be completely ruled out due to study design, we addressed this as best as possible in our statistical approach. Not only were relationships between statin use and cognitive outcomes independent of cardiovascular risk factors, they remained significant when controlling for coronary artery disease, long-term average LDL cholesterol concentration, *Apo E4* status, and two brain imaging measures known to affect cognitive performance. Furthermore, when incorporating the propensity score for statin use, statin use remained statistically significantly related to cognitive impairment, and to poor performance on memory tasks. Thus, based on our previous publication[18] and this study's results, we doubt that associations between statin use and poor cognitive outcomes are due merely to confounding by indication.

We examined statin class (lipophilic *vs* hydrophilic), a factor which may be an important consideration[31,38,39]. However, since almost all participants used lipophilic statins, analyses by statin class were not possible. Even though both classes of statins can cross the blood-brain barrier, lipophilic statins may accumulate in the brain more readily and/or rapidly than hydrophilic statins[39]. The exact nature of how statins affect the brain are unknown, and most of our knowledge is derived from animal or cell culture studies. Animal studies suggest that statins can exert negative impacts on both myelin[40-42] and neuronal health[2,3]. Other studies report neuroprotective effects of statins[43], while many studies show no effect (see reviews[6,44]). In addition, statins appear to promote cerebral angiogenesis at therapeutic doses, although angiostatic effects occur at higher concentrations[45].

Lastly, our study population differs from those of previous studies assessing statin use and cognitive function in two important ways: Our participants are middle-aged adults who were diagnosed with T1D in childhood, with a median duration of statin use of 6 years. This is in contrast to prior studies which primarily assessed relationships between statins and cognition in overall healthy, elderly adults aged 60 years and older, who used statins for only a short time; most previous cognitive studies examined statin use over periods of less than 3 wk to 1 year, although at least one study examined participants who used statins for 10+ years[5,6,46]. Moreover, these prior studies have not consistently shown evidence of a beneficial effect of statins on cognitive performance. In fact, the British Association for Psychopharmacology recently stated that "until further evidence is available, ...statins (among other drugs)… cannot be recommended either for the treatment or prevention of Alzheimer's disease"[47].

Why are these differences important? First, our participants have been exposed to metabolic dysregulation since childhood, a crucial period of brain development. This might make them more vulnerable to

negative consequences of statin therapy than would occur in people without T1D; if diabetes in childhood limited cerebral gray or white matter development, as brain imaging studies suggest, then these individuals may be less able to compensate for statin-related insults to the brain. Second, myelination occurs into early adulthood, with an additional "late wave" of myelination occurring during the 4th decade of life[48]. Exposure to statins during this time may negatively impact the myelination process, and these effects may be most noticeable in people with chronic diseases that negatively impact cerebral white matter development, as appears to occur in people with childhood-onset T1D[10]. Third, most prior studies were conducted in populations with much shorter exposure to statins than our participants have experienced. This is important because statins appear to promote glial progenitor cells to differentiate into oligodendrocytes, accompanied by a loss of uncommitted glial progenitor cells[16]. Thus, initiation of long-term statin use by middle-age, as is recommended for T1D patients, may reduce the pool of progenitor cells for future recruitment, thus making these patients less resilient to cerebral insults from normal aging or T1D-related vascular damage. This, in turn, may contribute to an increased risk for cognitive impairment in this vulnerable patient population.

These results, while compelling, need to be replicated before considering changes in how to best manage lipid profiles and cardiovascular risk in T1D. Limitations of the study include that study design does not allow us to test whether statin use preceded the onset of cognitive impairment. We cannot assess whether cessation of statin treatment would lead to improved cognitive function, particularly on memory tasks, because this is an observational study. Even though T1D duration was not related to cognitive impairment, these results may not be generalizable to middle-aged adults with adult-onset T1D, as such individuals are not exposed to diabetes-related metabolic disturbances during childhood, a critical window of brain development. Strengths of our study include a well-characterized T1D cohort with 25 years of risk factor data, use of an extensive neuropsychological test battery to assess multiple cognitive domains, and inclusion of brain imaging markers known to correlate with cognitive performance.

Identifying modifiable risk factors for cognitive impairment in T1D is an important public health concern because cognitive impairment may negatively impact these individuals' ability to adhere to their diabetes management regime, ultimately leading to higher healthcare costs, increased rates and/or severity of diabetes-related complications, disability, and quality of life issues. It is premature to make decisions about statin use in the management of cardiovascular risk in T1D based solely on the current study findings. At the same time, we encourage clinicians to engage their T1D patients in open dialog to address any concerns over perceived changes in cognitive function.

ACKNOWLEDGMENTS

We would like to thank the individuals with type 1 diabetes from the Pittsburgh Epidemiology of Diabetes Complications Study for their continued participation in this on-going study.

COMMENTS

Background

Type 1 diabetes (T1D) negatively affects cognitive function, but the risk factors contributing to cognitive impairment remain to be elucidated. This is particularly true for middle-aged and older adults living with diabetes since childhood and who are also experiencing the effects of advancing age on cognitive function.

Research frontiers

Statins are routinely prescribed for primary and secondary prevention of coronary events in people with T1D. Despite the on-going controversy regarding whether statins negatively impact cognitive function, especially the memory domain, there is a lack of data examining statins as a risk factor for cognitive impairment in this patient population.

Innovations and breakthroughs

As compared to never-statin users, statin use was related to greater odds of cognitive impairment. In addition, statin use was significantly related to lower performance on memory tasks. These relationships were robust to adjustment for coronary artery disease, long-term low density lipoprotein cholesterol levels, ApoE status, education, and age. Confounding by indication was also addressed using propensity score analysis.

Applications

Initiation of long-term statin use by middle-aged adults with childhood-onset T1D may negatively affect cognitive function, with strongest effects on memory. These results should be investigated in other T1D populations, preferentially in longitudinal studies with cognitive assessments and brain imaging assessed pre- and post- statin exposure.

Terminology

White matter hyperintensities are non-specific brain imaging markers of cerebral small vessel disease and are highly correlated to cognitive impairment and depression in adults ages 65 and older. Different visual rating scales are used to classify their severity; the authors chose the Fazekas scale, with "1" indicating mild white matter hyperintensities, and "2" or "3" indicating moderate to severe white matter hyperintensities.

Peer-review

This paper aims to test the correlation between statin use and cognitive impairment in adults with childhood-onset T1D, as a group of patients with chronic exposure to metabolic dysregulation. It is a valuable study, and the results are well analyzed.

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ORIGINAL ARTICLE

Observational Study

Risk factors for low high-density lipoprotein among Asian Indians in the United States

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Author contributions: All authors contributed equally to this work, read and approved the final manuscript; Misra R and Patel TG designed the research and completed the data collection; Misra R supervised the project, carried out the data analysis and drafted/edited the manuscript; Lucke-Wold B completed the literature review, created the tables and drafted/edited the manuscript; Patel TG drafted and edited the manuscript.

Institutional review board statement: The study was approved by the institutional review board of two universities, for data collection and for data analysis (de-identified) for this current manuscript by West Virginia University.

Informed consent statement: Participation was voluntary, and informed consent was obtained from all subjects prior to participation. The study was approved by the institutional review board of Texas AM University. In order to protect anonymity, unique participant codes were created based on initials of first and last name and numbers for each participant.

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

Data sharing statement: The DIA study used an 18-page survey to assess various constructs and anthropometric and clinical data to assess prevalence and risk for diabetes. Clinical information and demographic questions pertaining to this study are referenced in the paper; details were also provided in the method section. The authors do not wish to share their data in such repositories because of the unique nature of this only large scale population-level data on immigrant Asian Indians in the US. However, the authors are willing to provide additional supporting files (in SPSS) on which the conclusions of the manuscript have been based.

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Abstract

AIM

To examine the differences in metabolic risk factors (RFs) by gender in the Asian Indian (AI) population in the United States.

METHODS

Using cross-sectional data from 1038 randomly selected Asian Indians, we investigated the relationship between metabolic syndrome (MetS) RFs, cardiovascular disease,

and diabetes.

RESULTS

A greater percent of women in this group had increased waist circumference and low high density lipoprotein (HDL) levels than men, but AI males had increased blood glucose, increased blood pressure, and increased triglycerides compared to females. Those individuals who met the MetS criteria had increased cardiovascular disease. One of the biggest single RFs for cardiovascular disease and diabetes reported in the literature for AIs is low HDL.

CONCLUSION

Our results show that lack of knowledge about diabetes, low physical activity, increased body mass index, and age were the factors most significantly correlated with low HDL in this population. Future studies and prospective trials are needed to further elucidate causes of the MetS and diabetes in AIs.

Key words: Asian Indians; Diabetes; Cardiovascular disease; Metabolic syndrome; Low high density lipoprotein

Core tip: Low high density lipoprotein (HDL) in American Indians is a significant risk factor for the metabolic syndrome. In particular, women with lack of knowledge about diabetes, decreased physical activity, and who have an increased body mass are at increased risk of low HDL.

Lucke-Wold B, Misra R, Patel TG. Risk factors for low high-density lipoprotein among Asian Indians in the United States. *World J Diabetes* 2017; 8(6): 297-303 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i6/297.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i6.297

INTRODUCTION

South-Asians who live in the United States have an increased risk for developing the metabolic syndrome (MetS)[1]. A proposed reason is poor dietary habits consistent with sedentary western lifestyle[2]. Few studies have looked at this unique population, but data from the Indian Americans national study suggests that Asian Indians (AIs) may be more susceptible to certain components that make up the MetS[3]. For example, Vasudevan and colleagues found that obesity was prominent in people from South-Asian descent but was often times underdiagnosed[4]. Furthermore, AIs have increased risk for cardiovascular disease due to genetic predisposition and low high density lipoprotein (HDL)[5]. What is unknown however is if gender plays a significant role in increasing susceptibility for the MetS in this population. Furthermore, it is unknown what individual components of the MetS are more closely associated with diabetes in this population.

In this paper, we investigate these important questions utilizing data collected from a cross-sectional survey in the United States. Although causative factors cannot be determined, this study provides valuable insight into the differences observed between genders in relation to individual components of the MetS. More importantly, it highlights, which components of the MetS are more closely correlated with diabetes in this population. Previous studies in India have found that women have lower HDL than men and that cardiovascular risk factors (RFs) such as diabetes, hypertension, and smoking are highly prevalent[6,7]. Herein we report that a greater percent of AI males have increased fasting glucose, blood pressure, and triglycerides compared to females and that a greater percent of AI females have increased waist circumference and decreased HDL compared to males.

MATERIALS AND METHODS

Sample and data collection

The sample consisted of 1038 randomly selected AIs aged ⩾ 18 years from seven United States cities (Houston, TX; Phoenix, AZ; Washington, DC; Boston, MA; San Diego, CA; Edison, NJ and Parsippany, NJ); sampling frame and data collection methodology was previously reported[8]. All participants consented for the study prior to completing phone interviews and subsequent anthropometric/fasting blood work. In order to protect anonymity, participant codes were created based on letter codes and numbers unique to each participant. Non-participants did not differ in gender, educational level, family history of diabetes and cardiovascular disease, or smoking status, but were significantly older than participants. Survey data were collected *via* telephone interviews by trained, multilingual AI staff; the response rate was 37%. All participants completed blood work (after a 10 h fast) and anthropometric measurements. Blood samples were centrifuged to separate plasma or serum, and shipped on ice to three core laboratories for biochemical analysis (Atherotech Laboratory (Birmingham, AL), Diabetes Diagnostic Laboratory (Columbia, MO), and Translational Metabolism Unit, Baylor College of Medicine (Houston, TX)). The Institutional Review Board of Texas A and M University approved the study.

Measures

Demographic information: Demographic information included age, gender, marital status, education, income, and access to conventional health care (health insurance coverage). Income was assessed as a categorical variable with response options ranging from < $10000 to ⩾ $150000. Body mass index (BMI) was calculated from height and weight (kg/m^2).

Knowledge of MetS RFs: Knowledge of 11 MetS RFs (age, high cholesterol, DM, male gender, menopause,

fat intake, overweight/obesity, family history, sedentary lifestyle, smoking, and stress) was assessed. Response options for each RF were 0 = no and 1 = yes. A MetS knowledge score was computed by summing the number of correct answers (Cronbach's α = 0.78); a higher score indicated greater knowledge.

Fasting glucose: Fasting capillary glucose (mg/dL) was measured using Accucheck Advantage (Roche Diagnostics, Indianapolis, IN). Although fasting serum glucose was collected and stored, analysis indicated abnormal levels with large standard deviations from the capillary glucose for one-third of the respondents. Hence fasting capillary glucose was used for calculating CMetS in the current analysis.

RFs for MetS: Plasma samples were assayed for TG, HDL using the vertical auto profile test at the Atherotech Laboratory (Birmingham, AL) as described previously[9]. The LDL-R subfraction was determined by subtracting Lp(a) and IDL from total LDL.

Waist circumference: For males/females, the cut-off of WC = 35.4/31.5 inches was used to define elevated WC in this study, based on the IDF criteria for South Asians; also it has a high sensitivity (0.901/0.923) and specificity (0.836/0.768) for identifying South Asians with BMI $\geqslant$ 25 kg/m^2[10].

Definitions

MetS: MetS was assessed according to the Harmonization criteria, *i.e.*, presence of $\geqslant$ 3 of the MetS RFs: central obesity, elevated triglycerides ($\geqslant$ 150 mg/dL), low HDL (< 40 mg/dL in males, < 50 mg/dL in females, or specific treatment for these lipid abnormalities), elevated BP ($\geqslant$ 130/$\geqslant$ 85 mmHg or treatment of previously diagnosed hypertension), and elevated fasting glucose ($\geqslant$ 100 mg/dL or previously diagnosed type 2 diabetes). Central obesity was defined as ethnicity-specific elevated WC (for South Asians: $\geqslant$ 35.4 inches for males, $\geqslant$ 31.5 inches for females, where South Asian included Chinese, Malay and Asian-Indian populations)[11].

Diabetes: Diabetes was defined as fasting blood glucose $\geqslant$ 126 mg/dL or a self-report of previously diagnosed diabetes. Impaired fasting glucose was defined as fasting blood glucose between 100-125 mg/dL.

Statistical analysis

All analyses were performed using IBM SPSS version 24.0 (Chicago, IL) by the authors. The statistical analysis was reviewed by an expert biostatistician at WVU for adequacy, appropriateness, homogeneity of the data including missingness prior to the multivariate analysis. Basic descriptive statistics were obtained for demographic variables and MetS RFs. Analysis of variance was used to examine the difference in MetS RFs by gender for the total sample and by those with type 2 diabetes mellitus (T2DM). The acceptance level for statistical significance was α = 0.05. Multiple logistic regression analysis was used to predict low HDL controlling for traditional RFs such as age, gender, BMI, lifestyle behaviors, family history of chronic diseases, and MetS knowledge, and diabetes status. Sample size calculations indicated that 656 participants would provide over 80% power to detect important differences in HDL risk.

RESULTS

MetS variables

Lauderdale *et al*[12] found that the MetS was significantly higher at all BMIs in Asian Americans *vs* non-Hispanic Whites. In those surveyed for our study, 62.7% had elevated fasting blood glucose (651/1038), 28.7% had elevated blood pressure (298/1038), and 41.3% had elevated triglycerides (429/1038). Sixty percent had an elevated waist circumference (623/1038) and 35.9% had low levels of HDL cholesterol (373/1038). These values were similar to those reported by Misra *et al*[13] from AIs living in northern California.

Variables by gender

Recent evidence indicates that AIs have genetic single nucleotide polymorphisms that make them susceptible to developing the MetS in the context of poor diets and western sedentary lifestyles[14]. What has not been adequately investigated however is the role of gender as increasing risk for metabolic criteria. We report in Table 1 significant differences between male and females for individual components of the MetS. Interestingly, males were more likely to have elevated blood glucose, elevated blood pressure, and elevated triglycerides compared to females whereas females were more likely to have elevated waist circumference and low HDL cholesterol.

Heart disease and T2DM

Trude *et al*[15] reported that AIs had a 7.8% prevalence of cardiovascular disease. In our cohort, we found that 7.2% of survey participants had been diagnosed with cardiovascular disease. MetS can increase the risk for cardiovascular disease and subsequent adverse outcomes[16]. Similarly, diabetes is increasingly prevalent in this population. Seventeen point four percent of survey participants had diabetes and 32.9% of participants had pre-diabetes. Like cardiovascular disease, diabetes increases the risk for long-term morbidity and mortality[17].

Diabetes and individual components of the MetS

Anjana *et al*[18] found that low HDL in Indians is a predictor for the progression to T2DM. In Table 1, we report that a greater percentage of AI females had low HDL than males. In Table 2, we look specifically at participants with diagnosed or undiagnosed T2DM. The majority (60.71%) of females diagnosed with diabetes

Table 1 Significant differences between male and females for individual components of the metabolic syndrome

Variable	% Male Meeting Criteria	% Female Meeting Criteria	Significance
Elevated blood glucose: ⩾ 100 mg/dL or previous diagnosis of diabetes	71.77%	59.69%	(F1, 971) = 15.64 P < 0.001
Elevated blood pressure: ⩾ 130/⩾ 85 mmHg or previous diagnosis of hypertension	38.32%	31.45%	(F1, 836) = 4.16 P = 0.042
Elevated triglycerides: ⩾ 150 mg/dL	48.65%	33.49%	(F1, 1011) = 23.65 P < 0.001
Elevated Waist Circumference: ⩾ 35.4 inches for males, ⩾ 31.5 inches for females	56.41%	67.78%	(F1, 1018) = 13.59 P < 0.001
Low HDL: Low HDL < 40 mg/dL in males, < 50 mg/dL in females or previous treatment for low HDL	34.48%	42.30%	(F1, 987) = 6.26 P = 0.012

HDL: High density lipoprotein.

Table 2 Specifically at participants with diagnosed or undiagnosed type 2 diabetes mellitus

Variable	% Males with diagnosed Diabetes who Meet the Criteria	% Female with diagnosed Diabetes who Meet the Criteria	Significance
Elevated blood pressure: ⩾ 130/⩾ 85 mmHg or previous diagnosis of hypertension	54.95%	54.72%	(F1, 162) = 0.001 P = 0.977
Elevated triglycerides: ⩾ 150 mg/dL	57.63%	51.72%	(F1, 174) = 0.545 P = 0.462
Elevated Waist Circumference: ⩾ 35.4 inches for males, ⩾ 31.5 inches for females	80.00%	80.70%	(F1, 175) = 0.012 P = 0.913
Low HDL: Low HDL < 40 mg/dL in males, < 50 mg/dL in females or previous treatment for low HDL	39.32%	60.71%	(F1, 171) = 7.185 P = 0.008

HDL: High density lipoprotein.

had low HDL compared to only 39.32% of males. Hence, further investigation is warranted to determine if low HDL in females might contribute to the onset and progression of diabetes in this population.

Multivariate analysis of RFs for low HDL

Mani *et al*[19] found that low HDL is a primary predictor of progression towards diabetes and the MetS. We were interested in what factors were significantly associated with low HDL in our AI population (Table 3). A significant effect was found for age of respondent (P = 0.03), physical activity level (P = 0.014), knowledge about diabetes risk factors (P = 0.017), and the BMI (P = 0.01). Other groups have shown similar correlations between these risk factors and low HDL in other populations with high prevalence of the MetS[20].

DISCUSSION

Cardiovascular disease mortality is significantly higher among AIs and MetS, a proxy to predict the development of CVD, is of concern in this high-risk group. A study in South Asia recently found that ethnic Chinese had the lowest incidence of the MetS whereas ethnic Indians had the highest rate[21]. The MetS in this population can lead to an increase in vascular inflammatory markers such as C reactive protein, which can accelerate the progression of T2DM[22]. Indians as a whole have increased risk for developing diabetes due to genetic predisposition for poor insulin secretion[23]. This predilection for diabetes is even present in AIs with low body weight[24]. Little is known about gender differences in individual MetS components among AIs; our results highlight significant differences among male and female AIs for the five MetS criteria in a large Asian Indian sample in the United States. The results have broad-reaching implications for public health education, primary prevention, and improving unhealthy behavior.

A greater percentage of AI males had increased blood glucose, increased blood pressure, and increased triglycerides compared to females. Interestingly however, females had increased waist circumference and lower HDL compared to males. Recent evidence suggests that low HDL has a strong genetic component and can significantly increase the risk for cardiovascular disease, diabetes, and mortality[25]. Although this study is cross-sectional in design, it does show an important finding in that AI women with diabetes are much more likely to have low HDL than males.

Our study results showed that the obesity was associated with low HDL levels among AIs. One plausible explanation might be the westernized lifestyle adapted upon immigration or acculturation to the United States society. In addition, the increased vulnerability to metabolic diseases including the MetS may be due to the unique body composition, which is marked by increase in abdominal obesity and percent body fat. This is termed the "Yudkin Yajnik paradox" where AIs with low BMI have higher percent body fat than African Americans and Europeans increasing their risk for metabolic diseases[26]. The typical AI phenotype is one of higher percent body fat, higher truncal, sub-cutaneous, and intra-abdominal fat, and less lean body mass[27]. These features are even noted in AI neonates[28]. These genetic findings coupled with biochemical indicators such as high levels of inflammatory markers, low levels of adiponectin, the co-existence of hyperinsulinemia, insulin resistance, hypertriglyceridemia, abnormal lipid profiles, endothelial dysfunction and hyperhomocysteinemia

Table 3 Significant factors associated with low high density lipoprotein in Asian Indian population

	Variables in the Equation							
		B	SE	Wald	df	Sig.	Exp (B)	95%CI for EXP(B)
								Lower / Upper
Step 1[a]	Diab_Category			0.229	2	0.892		
	Diab_Category(1)	-0.089	0.341	0.068	1	0.795	0.915	0.469 / 1.787
	Diab_Category(2)	0.057	0.252	0.051	1	0.821	1.059	0.646 / 1.737
	Gender(1)	-0.294	0.236	1.562	1	0.211	0.745	0.470 / 1.182
	Age of respondent	0.028	0.010	8.793	1	0.003	1.029	1.010 / 1.048
	Physicalact	1.004	0.409	6.034	1	0.014	2.730	1.225 / 6.082
	Nutrition	0.100	0.407	0.061	1	0.806	1.105	0.498 / 2.453
	TobaccoUse_Rec(1)	-0.513	0.508	1.019	1	0.313	0.599	0.221 / 1.621
	FamHistory	0.148	0.226	0.425	1	0.515	1.159	0.744 / 1.807
	Income_Rec			3.456	2	0.178		
	Income_Rec(1)	0.451	0.305	2.180	1	0.140	1.570	0.863 / 2.857
	Income_Rec(2)	0.424	0.254	2.801	1	0.094	1.529	0.930 / 2.513
	Lifestyle	-0.389	0.258	2.268	1	0.132	0.678	0.408 / 1.124
	Knowledge of CVD risk	0.099	0.067	2.191	1	0.139	1.104	0.968 / 1.259
	Knowledge of DM risk	-0.230	0.097	5.652	1	0.017	0.795	0.658 / 0.960
	BMI_Up	-0.077	0.03	6.560	1	0.010	0.926	0.873 / 0.982
	Constant	2.182	1.194	3.338	1	0.068	8.864	

[a]Variable(s) entered on step 1: Diab_Category, gender, age of respondent, physicalact, nutrition, TobaccoUse_Rec, FamHistory, Income_Rec, Lifestyle, Knowledge of CVD risk, Knowledge of DM Risk, BMI_Up. CVD: Cardiovascular disease; BMI: Body mass index; DM: Diabetes mellitus.

set the stage for chronic low grade inflammation that exacerbates morbidity and mortality among AIs. Since muscle mass is an indicator of insulin sensitivity, a lower muscle mass re-routes energy from large carbohydrate meals typical in the Asian Indian diet into hepatic lipogenesis compromising muscle glycogen synthesis. The outcome is atherogenic dyslipidemia[29], a menacing combination that is metabolically linked to insulin resistance, promotes sub-clinical chronic inflammation, and is strongly associated with type 2 diabetes and cardiovascular disease. Underlying genetic factors such as gene variants and polymorphisms further exacerbate the risk for AIs. These factors include the ectonucleotide pyrophosphate phosphodiesterase 1 121Q variant implicated in negatively influencing insulin receptor signaling[30], the *DOK5* gene[31] that increases the risk for diabetes in immigrant AIs[30], apolipoprotein E gene polymorphisms and the Myostatin gene linked to abdominal obesity[32], the AMDI variant in homocysteine metabolism that predisposes children to obesity[33], and finally the PPAR-gamma polymorphisms that contribute to non-alcoholic fatty liver disease[34]. Research shows low HDL level is a strong and independent risk factor for cardiovascular disease[35]. A meta-analysis showed one mg/dL increase of HDL-C levels is associated with a 2%–3% decreased CVD risk[36]. It may be one of the primary reasons why AIs are disproportionately burdened by coronary artery disease at younger ages and in more severe forms[37,38]. Our results concur with prior studies that AIs have amplified low levels of HDL as compared to Non-Hispanic Whites and Europeans. Furthermore, the higher prevalence of low HDL among AI females (61%) than AI males in this study (39%) support prior literature on a higher prevalence among AI women ranging from 65%-79%[37,39,40] than among AI men 35%-67%[37,39-41].

Low HDL can be exacerbated in the context of sedentary habits and a poor diet in Indian populations[42]. We found that obesity, decreased physical activity, lack of knowledge about diabetes, and advanced age are significantly associated with low HDL in this population. AIs who have lived in the United States for greater than 10 years are more likely to have a sedentary lifestyle, increased obesity, and increased risk of diabetes type 2[43]. Ghai *et al*[44] compared an AI cohort to a White non-Hispanic cohort. They noted that AIs were less likely to eat 5 servings of fruit and vegetables a day and less likely to engage in physical activity. They were more likely however to have a lower calorie diet, not smoke, and not consume alcohol[44]. The authors concluded that genetics in addition to lifestyle factors contributed to the development of the MetS in AIs.

Going forward, it will be important to isolate key genetic components that increase the susceptibility of low HDL in AI women. Once these components are identified, it will be possible to develop a tailored treatment approach and education with personalized medicine. Additionally, public health initiatives can provide an important element for training individuals to engage in health promoting behavior. Diabetes prevention and management programs can help individuals learn key skills on how to prevent and manage the MetS. These programs can be especially influential for the AI population[45].

The MetS affects the AI population and may contribute to the increased prevalence of diabetes. Interestingly, we found a significant difference in metabolic components between men and women. A greater percent of women met the waist circumference and low HDL criteria than men. Furthermore, in this cohort we found a high prevalence of diagnosed cardiovascular disease, which has been linked to increased adverse vascular events. Understanding the genetic and environ-

mental components that contribute to the increased MetS in this population will be essential in order to improve and tailor public health and pharmacologic treatment approaches.

COMMENTS

Background

American Indians are prone to develop the metabolic syndrome (MetS) once they adapt a western lifestyle.

Research frontiers

This article addresses the importance of low high density lipoprotein for the development of diabetes and the MetS in American Indians.

Innovations and breakthroughs

Improving education about the MetS for this population will be beneficial.

Applications

In particular, diabetes prevention and management programs will be highly important to implement for this population.

Terminology

The authors specifically focused on the components of the MetS.

Peer-review

The authors utilized data collected from a cross-sectional survey from 1038 randomly selected Asian Indians in the United States to investigate the relationship between metabolic syndrome risk factors, cardiovascular disease, and diabetes. The article implicates that one of the biggest single risk factors for cardiovascular disease and diabetes reported in the literature for Asian Indians is low high density lipoprotein. It is suitable to the Journal and could be helpful in clinic application.

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ORIGINAL ARTICLE

Observational Study

Interleukin-18 polymorphism as an inflammatory index in metabolic syndrome: A preliminary study

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Abstract

AIM

To assess circulatory levels of interleukin-18 (IL-18) and determine whether the presence of IL-18 promoter polymorphism influences metabolic syndrome phenotypes.

METHODS

This study recruited one hundred and eighty individuals divided into three groups with sixty subjects each as: Normal weight (18.0-22.9 kg/m^2), overweight (23.0-25.9 kg/m^2) and obese (> 26.0 kg/m^2) according to South Asian criteria of BMI. Fasting blood glucose (FBG), Lipid profile, insulin, IL-18 and tumor necrosis factor (TNF)α were measured using ELISA kits, whereas low density lipoprotein (LDL)-cholesterol, insulin resistance (HOMA-IR) and insulin sensitivity (QUICKI) were calculated. The body fat percentage (BF) was measured through bioelectrical impedance analysis; waist and hip circumference were measured. Genotyping of IL-18 -607 C/A polymorphism was performed by using tetra-primer amplification refractory mutation system. Student *t* test, One-way analysis of variance, Hardy-Weinberg equilibrium, Pearson's χ^2 test and Pearson's correlation were used, where a P value < 0.05 was considered significant.

RESULTS

In an aged matched study, obese subjects showed higher levels of FBG, cholesterol, triglycerides and LDL levels as compared to normal weight (P < 0.001).

Highest levels of IL-18 and TNF levels were also seen in obese subjects (IL-18: 58.87 ± 8.59 ng/L) (TNF: 4581.93 ± 2132.05 pg/mL). The percentage of IL-18 -607 A/A polymorphism was higher in overweight and obese subjects *vs* normal weight subjects ($P < 0.001$). Moreover, subjects with AA genotype had a higher BF, insulin resistance, TNFα and IL-18 levels when compared with subjects with AC (heterozygous) or CC (wild type) genotypes. However, we did not find any difference in the lipid profile between three subgroups.

CONCLUSION

This preliminary data suggests that IL-18 polymorphism affects IL-18 levels that might cause low grade inflammation, further exacerbated by increased TNFα. All these increase the susceptibility to develop MetS. Further studies are required to validate our findings.

Key words: Metabolic syndrome; Interleukin-18; Polymorphism; Obesity; Body fat; High density lipoprotein; Low density lipoprotein; Insulin

Core tip: Interleukin-18 (*IL-18*) gene polymorphisms may influence the expression of its levels. This in turn increases the risk of metabolic syndrome (MetS). Therefore, we aimed to assess the circulatory levels of IL-18 and determine whether the presence of IL-18 promoter polymorphism influences MetS phenotypes. Subjects with AA genotype had a higher body fat, insulin resistance, tumor necrosis factor α and IL-18 levels when compared with subjects with AC (heterozygous) or CC (wild type) genotypes. This preliminary data suggests that IL-18 polymorphism affects IL-18 levels that might cause low grade inflammation. All these increase the susceptibility to develop MetS. Further studies are required to validate our findings.

Fatima SS, Jamil Z, Abidi SH, Nadeem D, Bashir Z, Ansari A. Interleukin-18 polymorphism as an inflammatory index in metabolic syndrome: A preliminary study. *World J Diabetes* 2017; 8(6): 304-310 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i6/304.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i6.304

INTRODUCTION

Interleukin-18 (IL-18), also known as interferon-gamma inducing factor, is a pro-inflammatory cytokine that belongs to the IL-1 superfamily. It is not only produced by immune cells like macrophages but is also express by keratinocytes, osteoblasts cells, pituitary gland and adrenal cortical cells[1]. IL-18 serves as a mediator of immune response by stimulating T-helper cells (Th-1) against infections[2]. In healthy individuals, its production by the host is in line to utilization as a defense and healing mechanism, maintained in a fine balance. However, it has been established that its over-production results in autoimmune inflammatory disorders[3]. Apart from its role in inflammation, IL-18 has also been associated with increased visceral adiposity and obesity. Studies report abnormally elevated circulating IL-18 levels in obese individuals while reduction in body weight is found to results in a concomitant reduction in IL-18 levels, supporting the fact that reduction in adipose tissue leads to a decline in secretion of pro-inflammatory cytokines[4]. Furthermore, IL-18 mediated inflammation is associated with cardiovascular disorders suggesting its role in atherosclerotic diseases[5]. In the context of hyperglycemia, there are certain oxidative mechanisms that increase circulating cytokines including IL-18, thereby linking high blood glucose to the pro-inflammatory cytokines[6]. Various studies have reported elevated serum IL-18 in patients with type 2 diabetes[7].

As the presence of risk factors for cardiovascular disease and type 2 diabetes mellitus increases the threat of developing metabolic syndrome (MetS), IL-18 has been implicated to play a critical role in such conditions[8]. In terms of polymorphism in the IL-18 gene, various SNP are reported in association with diseases like type 1 diabetes[9], chronic hepatitis B virus infection[10], asthma[11].

The *IL-18* gene is located on chromosome 11 (11-q22.2-22.3), and contains many polymorphism, especially in the promoter region[12]. One such polymorphism is the -607 A/C that seems to affect the expression level of IL-18 at transcription level[13]. In addition, it has been associated with the development of cardiovascular disease such as vascular endothelial damage and formation of atherosclerosis[14]. The relationship between the promoter region polymorphism of IL-18 and MetS phenotypes is scarce. Therefore, we aimed to assess the circulatory levels of IL-18 and determine whether the presence of IL-18 promoter polymorphism influences MetS phenotypes.

MATERIALS AND METHODS

Patient recruitment

This cross-sectional study recruited 180 healthy male individuals from the waiting areas of outpatient department of Aga Khan University. The subjects were divided into three groups. Group A: normal weight (18.0-22.9 kg/m^2), Group B: overweight (23.0-25.9 kg/m^2), Group C: obese (> 26.0 kg/m^2) according to South Asian criteria of BMI[15]. Subjects with diabetes mellitus, hypertension [resting blood pressure (BP) 170/100 mmHg], dyslipidemia, body weight fluctuation of 5 kg in the recent 6 mo, smokers, alcoholics, any acute illness during last one month, as well as those taking anti-inflammatory medications were excluded. This study was approved by the institutional ethical committee and all participants gave a written and informed consent to participate in this study. The sample size was calculated

in order to achieve 80% power to detect an odds ratio of at least 2 among obese, with a two sided alpha value of 95% (NCSS/PASS version 11 software for power analysis and sample size).

Anthropometric data

The weight and height of all the subjects were measured in kilograms and meters respectively, using a weight scale with a built-in Stadiometer (ZT-120 Health Scale, Nanjing Everich China). Waist circumference and hip circumference was measured using the WHO protocol[16]. Subjects were asked to stand in an erect posture wearing light clothing. BMI was calculated by dividing weight by height squared (kg/m^2)[17]. While body fat percentage was measured using Diagnostic Scale BG55 (Beurer Germany) through bioelectrical impedance matching/analysis.

Biochemical profile

Six milliliter of blood was collected from the study participants after an overnight fast of 12 h. Fasting plasma glucose and Lipid profile were measured using commercially available kits as per the vendor's instruction (Merck, France). Low density lipoprotein (LDL)-cholesterol levels were calculated using the Friedewald equation[18]. Fasting insulin, IL-18 and TNFα levels were measured using an ELISA kit (DIA source Immuno Assay S.A., Belgium). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) index [fasting insulin (units per milliliter) × fasting glucose milligram/deciliter)/405][19], and insulin sensitivity was calculated by (QUICKI) {1/[log(fasting insulin) + log(fasting glucose)]}[20].

Genotyping

DNA extraction was performed using commercially available Qiagen DNA extraction kit (Cat. #51185, Valencia, CA United States). Genotyping of IL-18 -607 C/A polymorphism was performed by using tetra-primer amplification refractory mutation system (TARMS-PCR) using the GoTaq® Hot Start Green Master Mix (Cat. # M5122, Promega Corporation, United States) as per the manufacturer's instructions with the following cycling conditions for PCR: 1 cycle for 5 min at 95 ℃ for initial denaturation followed by 35 cycles at 95 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 30 s followed by a final extension of 10 min at 72 ℃. PCR products were electrophoresed in 2% agarose gel. Genotyping quality control was performed in 10% of the samples by duplicate checking (rate of concordance in duplicates was > 99 %). Tetra arms primers used for amplifying IL-18 -607 (C/A) were as follows: Control Band (Outer forward: CCTACAATGTTACAACACTTAAAAT; Outer reverse: ATAAGCCCTAAATATATGTATCCTTA) (product size 440 bp); A allele [Inner forward: GATACCATCATTAGAATTTTGTG (product size 278 bp)] and C allele [Reverse inner GCAGAAAGTGTAAAAATTATCAA (product Size 208 bp)] (Figure 1). The study was approved by the institutional ethical review board (3597-BBS-ERC-15), and all subjects gave a written and informed consent.

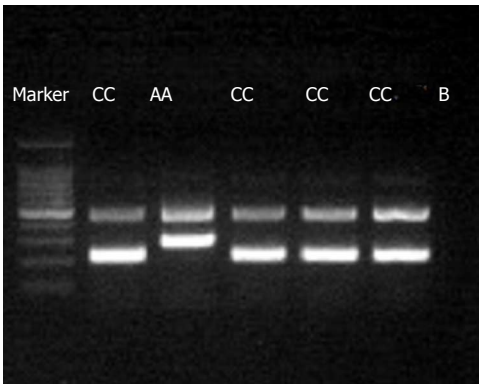

Figure 1 Genotype distribution of selected samples for the tetra arms PCR. Marker is 100 bp DNA ladder and B is blank.

Statistical analysis

A descriptive statistical analysis of continuous variables was performed using SPSS (version 21; SPSS Inc., Chicago, IL, United States). Data on continuous variables were calculated as mean ± SD, whereas data on categorical variables was presented as frequencies and percentages. Statistical comparisons were computed using a student *t* test, one-way analysis of variance (ANOVA) and Pearson's χ^2 test of independence. Pearson's correlation (r) were used to determine the correlation between serum IL-18 levels and lipid profile, fasting blood glucose, insulin and body fat parameters. Hardy-Weinberg equilibrium (HWE) was calculated for IL-18 SNP. Significance and effect size of minor allele with study parameters were determined under an additive model of inheritance. In all statistical analysis performed *P* values < 0.05 were considered significant.

RESULTS

Table 1 shows the biophysical and biochemical data of the study subjects. All three groups were aged matched, therefore no significant difference was observed amongst the groups. Considerable difference was observed in terms of raised BMI and BF in obese group as compared to controls ($P < 0.001$). Similarly, obese group had a higher FBG, insulin, cholesterol, triglycerides and LDL levels as compared to controls ($P < 0.001$). Interestingly no differences were seen in the high density lipoprotein (HDL) levels of our study groups. IL-18 and TNF levels showed an increasing trend from normal weight to obese, with the highest levels seen in obese group (IL-18: 58.87 ± 8.59) (TNF: 4581.93 ± 2132.05). We next performed the correlation of IL-18 levels with the study parameters and report a strong positive correlation of IL-18 with BMI, Waist circumference, FBG, insulin, HOMA-IR, QUICKI, Choles-

Table 1 Biophysical and biochemical data of the study subjects

Variables	Normal weight (18-22.9 kg/m^2) (n = 60)	Overweight (23-25.9 kg/m^2) (n = 60)	Obese (>26 kg/m^2) (n = 60)	P value
Age (yr)	26.21 ± 3.876	25.76 ± 4.059	27.30 ± 5.389	NS
BMI (kg/m^2)	20.48 ± 1.30	24.20 ± 0.91[1]	28.59 ± 3.34[1,2]	< 0.001
Body fat (%)	19.26 ± 7.17	28.70 ± 9.10[1]	34.89 ± 4.47[1,2]	< 0.001
Waist circumference (cm)	88.03 ± 10.53	88.46 ± 7.69	1.9.26 ± 11.47	< 0.001
Hip circumference (cm)	77.69 ± 11.78	84.43 ± 0.69	100.41 ± 12.89	< 0.001
WHR (cm)	0.87 ± 0.05	0.871 ± 0.054	0.91 ± 0.073	< 0.001
Fasting blood glucose (mg/dL)	89.31 ± 15.23	105.59 ± 12.50[1]	119.00 ± 25.71[1]	< 0.001
Insulin (uIU/mL)	20.48 ± 7.95	34.96 ± 4.47[1]	41.13 ± 7.81[1,2]	< 0.001
HOMA-IR	4.70 ± 2.66	9.05 ± 1.34[1]	11.88 ± 2.81[1,2]	< 0.001
QUICKI	0.31 ± 0.02	0.27 ± 0.00[1]	0.27 ± 0.01[1]	< 0.001
Cholesterol (mg/dL)	145.72 ± 30.08	147.44 ± 37.91	209.06 ± 55.51[1,2]	< 0.001
Triglyceride (mg/dL)	124.82 ± 43.92	137.58 ± 65.60	167.03 ± 55.99[1,2]	< 0.001
HDL(mg/dL)	39.96 ± 9.38	38.37 ± 8.00	36.40 ± 6.40	NS
LDL(mg/dL)	75.43 ± 30.03	79.57 ± 40.01	134.78 ± 58.99[1,2]	< 0.001
TNFα (pg/mL)	810 ± 1233	4455 ± 2390[1]	4581 ± 2132[1]	< 0.001
IL-18 (ng/L)	25.34 ± 6.57	41.96 ± 4.50[1]	58.87 ± 8.59[1,2]	< 0.001

Values are expressed as mean ± SD. Comparison between groups was tested by One way analysis of variance followed by Tuckey's *post hoc* test. [1]Statistically significant as compared to normal weight; [2]Statistically significant as compared to overweight. Significance set at P value < 0.05. HOMA-IR: Homeostasis model of insulin resistance; QUICKI: Quantitative insulin sensitivity check index; HDL: High density lipoprotein; LDL: Low density lipoprotein; TNF: Tumor necrosis factor; WHR: Waist to hip ratio.

Table 2 Correlation of interleukin-18 with metabolic syndrome phenotypes

Variable	Unadjusted r	Adjusted r (age, BMI and body fat %)
Age (yr)	0.302	--
BMI (kg/m^2)	0.751	--
Body fat (%)	0.518	--
Waist circumference (cm)	0.333	0.413
Hip circumference (cm)	0.695	-0.110[NS]
Fasting blood glucose (mg/dL)	0.559	0.376
Insulin (uIU/mL)	0.655	0.205[NS]
HOMA-IR	0.699	0.344
QUICKI	-0.600	-0.496
Cholesterol (mg/dL)	0.514	0.265
LDL(mg/dL)	0.464	0.245
TNFα	0.577	0.491

Pearson Correlation was applied. All associations remained significant after adjustment except insulin ([NS]non-significant), statistically significant P value < 0.01. HOMA-IR: Homeostasis model of insulin resistance; QUICKI: Quantitative insulin sensitivity check index; LDL: Low density lipoprotein; TNF: Tumor necrosis factor.

Table 3 Genotype frequency among study subjects

Genotype distribution				
	CC	AC	AA	P value
Normal weight	27	23	10	< 0.001
Overweight	11	25	24	
Obese	12	24	24	
Allele frequency				
	A allele	C allele	OR (95%CI)	P value
Normal weight	42 (35.0)	78 (65.0)	-	-
Overweight	47 (39.17)	73 (60.83)	2.81 (1.66-4.68)[1]	< 0.001
Obese	48 (40.00)	72 (60.00)	2.91 (1.72-4.94)[2]	

In all divisions the HWE was > 0.05. [1]Normal weight *vs* overweight and [2]normal weight *vs* obese calculated by Pearson's χ^2 test. Allele frequencies are given as absolute values with percentage given in parentheses.

terol, and TNFα while a moderate correlation was seen for LDL and BF. All these associations remained significant after multiple adjustments for confounding factors like age, BMI and BF, except insulin (Table 2 and Figure 2).

The genotype distributions was in accordance with HWE in total study subjects (P = 0.222) and in subgroups (Normal Weight: P = 0.281; Overweight: P = 0.663; Obese: P = 0.196). The percentage of IL-18 -607 A/A genotype was higher in overweight and obese subjects *vs* normal weight subjects (P < 0.001 Table 3). Moreover, subjects with AA genotype had a higher BF, insulin resistance, TNFα and IL-18 levels when compared with subjects with AC or CC genotypes. However, we did not find any difference in the lipids profile between three subgroups (Table 4).

DISCUSSION

IL-18 is pleiotropic cytokine acting in both acquired and innate immunity. Additionally it also acts to stimulate the production of TNFα[1] which is also a key player associated with higher BMI[21,22]. These cytokines in turn predispose an individual to develop MetS phenotypes.

In an age matched study group, we observed a positive association of serum IL-18 concentration with BMI, FBG, and serum TG, where body fat percentage contributed most to the variation of serum IL-18 concentration. Furthermore, our study shows circulating IL-18 levels were associated with measures of insulin resistance (HOMA-IR) and decreased insulin sensitivity (QUICKI) in apparently healthy obese subjects. Among other factors causing a rise in IL-18 levels, nutritional

Table 4 Stratification of study subjects according to genotype distribution

Variables	CC (n = 57)	AC (n = 72)	AA (n = 51)	P value
BMI (kg/m^2)	23.36 ± 4.05	24.60 ± 4.137	25.25 ± 3.35[1]	0.05
Body fat (%)	24.70 ±10.30	28.70 ± 8.59[2]	29.31 ± 9.46[1]	< 0.001
Waist circumference(cm)	92.78 ± 16.61	96.48 ± 13.21	97.32 ± 13.82	0.085
Hip circumference (cm)	74.18 ± 4.23	67.40 ±3.99	64.51 ± 4.84	0.694
WHR (cm)	0.88 ± 0.069	0.88 ± 0.061	0.89 ± 0.06	0.490
Fasting blood glucose (mg/dL)	103.41 ± 23.43	101.79 ± 21.01	110.32 ± 21.97	> 0.05
Insulin (uIU/mL)	28.83 ± 11.91	32.76 ± 11.39[2]	34.67 ± 9.61[1]	0.002
HOMA-IR	7.75 ± 4.34	8.44 ± 3.71	9.47 ± 3.00[1]	0.007
QUICKI	0.29 ± 0.02	0.28 ± 0.02	0.28 ± 0.01[1]	0.046
Cholesterol (mg/dL)	161.01 ± 39.01	165.12 ± 55.03	177.42 ± 60.72	> 0.05
Triglyceride (mg/dL)	135.66 ± 42.29	145.61 ± 61.45	146.78 ± 64.08	> 0.05
HDL(mg/dL)	38.29 ± 8.84	38.48 ± 56.32	37.17 ± 6.75	> 0.05
LDL(mg/dL)	88.99 ± 5.38 (SEM)	94.73 ± 6.63 (SEM)	107.77 ± 7.89 (SEM)	> 0.05
TNFα (pg/mL)	2521 ± 353.9 (SEM)	3403.08 ± 313.25 (SEM)	3760.35 ± 336.55[1] (SEM)	0.008
IL-18 (ng/L)	37.16 ± 19.35	43.00 ± 13.19[2]	46.311 ± 13.07[1]	0.001

[1]AA *vs* CC; [2]AC *vs* CC. HDL: High density lipoprotein; LDL: Low density lipoprotein; TNFα: Tumor necrosis factor alpha; IL-18: Interleukin-18.

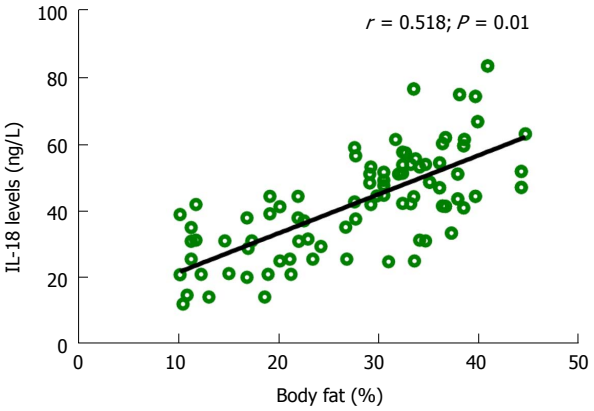

Figure 2 Correlation of interleukin-18 levels with body fat percentage. IL-18: Interleukin-18.

states, such as hyperglycemia, and fat mass increase[23] have been validated the most. In particular, adipocytes from obese individuals were found to secrete a threefold higher IL-18 *vs* lean ones[24], identifying an essential role of IL-18 in regulating fat distribution[25]. Though, an animal study demonstrated that deficiency of IL-18 resulted in obesity and insulin resistance in mice and the phenotype could be rescued by exogenous administration of IL-18[26].

We further evaluated the presence of a promote gene polymorphism in these subjects. Interestingly, we report that subjects with AA genotype had a higher BMI, BF, insulin resistance and IL-18 levels. Moreover, raised IL-18 increased the chances of developing of MetS in our study subjects (OR = 2.72, 95%CI: 1.28-5.74, P = 0.008). Results regarding the association of -607 SNP and MetS phenotypes are not consistent. One study reported a decreased proportion of A/A genotype in type 1 diabetic patients relative to control subjects[9]. However, another[27] found higher proportion of A/A genotype in type 1 diabetic patients but no risk association could be identified. Another study, conducted in Chinese population reported a higher proportion of A/A genotype in patients with type 2 diabetes, which is somewhat similar to our report. Therefore, it is empirical to identify the different genetic influences among different races when considering genotype data and risk association. For instance, A allele at position of -607 was a protective allele from type 1 diabetes in a Polish population[9], while in a population from United Kingdom, the significant association was not found[27].

These seemingly conflicting results suggested that IL-18 probably acts as a feedback signal for obesity, hyperglycemia, and positive energy balance. Alternatively, it might be a consequence of sensitivity in those subjects to the effect of IL-18. This may prove that inflammatory marker (IL-18) may just not be an indicator for chronic inflammation and obesity but has a role in pathway leading to MetS. Another outcome of our study was the relation of IL-18 with increased levels of cholesterol and LDL. This opens up another avenue about the role of IL-18 in atherosclerosis and presents a great opportunity to work further in this field. Some of previous studies have shown association of circulating IL-18 levels with cardiovascular mortality among patients with coronary artery disease[5]. However, contrasting data also exists which states no or weak association of IL-18 levels with BMI and lipid profile in European men[28], this may suggest that association may vary according to the population. In addition to Interleukin 18, recent repost suggested that other variants such as Interleukin-23/IL-17 axis has also been independently affiliated with obesity in women especially related to increase visceral fat, insulin resistance, and leptin levels[29] as well as in causing hypertension and increased cardiovascular risk[30].

One of the limitations of this study rests in the study-design. As this was a cross sectional study the association of IL-18 with all of these metabolic traits could not be established, further more we were unable to record the nonalcoholic fatty liver disease through

ultra sonographic analysis. Nevertheless our results clearly show that IL-18 can be used a marker for obesity and supports the hypothesis that IL-18 may be involved in pathway of MetS and form a link between metabolic risk factors, diabetes, and cardiovascular diseases specially in south Asian population.

COMMENTS

Background

Cytokines are implicated for causing lipid derangement and insulin resistance. Furthermore, polymorphisms in the interleukin-18 (*IL-18*) genes influences expression levels and may increase the risk of metabolic syndrome (MetS).

Research frontiers

The authors' results clearly show that IL-18 can be used a marker for obesity and supports the hypothesis that IL-18 polymorphism may be involved in pathway of MetS and form a link between metabolic risk factors, diabetes, and cardiovascular diseases specially in south Asian population. This can lead to precision treatments to reduce the burden of obesity.

Innovations and breakthroughs

The literature suggests a mixed role of *IL-18* gene polymorphisms in MetS or diabetes. However, the present study suggests a new role promter gene polymorphisms in modulating MetS phenotypes.

Applications

The authors' study provides a preliminary report of association of IL-18 levels and its polymorphism though at this stage no therapeutic role can be elucidated.

Terminology

MetS: It is a cluster of conditions such as diabetes, hypertension, increased waist circumference and lipid levels in in individual; Polymorphism: The presence of genetic variation within a population.

Peer-review

Preliminary results of this work are very interesting.

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EDITORIAL

PCSK9 and carbohydrate metabolism: A double-edged sword

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays a paramount role in the degradation of low-density lipoprotein (LDL) receptors (LDLR) on the hepatic cells surface and subsequently affects LDL particles catabolism and LDL cholesterol (LDL-c) levels. The anti-PCSK9 monoclonal antibodies lead to substantial decrease of LDL-c concentration. PCSK9 (which is also expressed in pancreatic delta-cells) can decrease LDLR and subsequently decrease cholesterol accumulation in pancreatic beta-cells, which impairs glucose metabolism and reduces insulin secretion. Thus, a possible adverse effect of PCSK9 inhibitors on carbohydrate metabolism may be expected by this mechanism, which has been supported by the mendelian studies results. On the other hand, clinical data have suggested a detrimental association of PCSK9 with glucose metabolism. So, the inhibition of PCSK9 may be seen as a double-edged sword regarding carbohydrate metabolism. Completed clinical trials have not shown a detrimental effect of PCSK9 inhibitors on diabetes risk, but their short-term duration does not allow definite conclusions.

Key words: Proprotein convertase subtilisin/kexin type 9; Diabetes; Carbohydrate metabolism; Low-density lipoprotein; Proprotein convertase subtilisin/kexin type 9 inhibitors

Core tip: Proprotein convertase subtilisin/kexin type 9 (PCSK9) may play a beneficial role in carbohydrate metabolism because it can decrease low-density lipoprotein receptor and subsequently decrease cholesterol

accumulation in pancreatic beta-cells, which impairs glucose metabolism and reduces insulin secretion. In contrast, clinical data have suggested a detrimental association of PCSK9 with glucose metabolism. These conflicting mechanisms may lead to a neutral effect on carbohydrate variables and explain the results of short-term clinical trials with PCSK9 inhibitors, which have not shown an increased diabetes risk.

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INTRODUCTION

Statins can dose dependently increase the incidence of new-onset diabetes mainly in patients with underlying abnormalities of carbohydrate metabolism. This effect is at least partially an "on target" effect related to the statin-induced inhibition of 5-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase[1-4]. These observations have led ongoing research to focus on the possible association of newer hypolipidemic drugs with incident diabetes. Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been identified as a key protein in lipid and lipoprotein metabolism, which plays a paramount role in the degradation of low-density lipoprotein (LDL) receptors (LDLR) on the hepatic cells surface and subsequently affects LDL particles catabolism and LDL cholesterol (LDL-c) levels (Figure 1)[5]. The anti-PCSK9 monoclonal antibodies bind circulating PCSK9, thus preventing PCSK9-induced degradation of LDLR. The administration of these drugs on top of conventional lipid lowering treatment substantially decreases LDL-c concentration by approximately 50% in various groups of high-risk patients, while the treatment is well tolerated[6]. Even though significant differences in the incidence of most adverse events were not observed between PCSK9 inhibitors-treated and placebo-treated patients, an increased incidence of neurocognitive events was observed, which needs further evaluation[7].

It has been shown that PCSK9 can decrease LDLR and subsequently decrease cholesterol concentrations in pancreatic beta-cells; thus, it may beneficially affect beta cell function, since the accumulation of cholesterol in beta-cells impairs glucose metabolism, reduces insulin secretion and can be associated with a diabetic phenotype[8]. Based on this concept, a crucial question emerges whether PCSK9 inhibitors can increase diabetes risk by inhibiting this beneficial effect (Table 1). This question is particularly relevant, because the results of genetic studies have shown contradictory results. Thus, even though no increased risk of diabetes or other changes in glucose homeostasis were found in individuals with PCSK9 loss-of function variants[9,10], carriage of the loss-of-function PCSK9 p.R46L mutation was associated with insulin resistance [increased homeostasis model assessment-insulin resistance (HOMA-IR) index] in those with apolipoprotein E_3/E_2 genotype[11]. However, another study did not confirm these results and showed that the p.R46L mutation was not associated with markers of glucose homeostasis, while p.R46L carriers did not experience an increased risk of new-onset diabetes mellitus[12]. Additionally, experimental data from animal models have also provided conflicting results. One study showed that PCSK9 deficiency does not alter insulin secretion and glucose tolerance in mice[13], while another study showed that PCSK9 deficient mice ($PCSK9^{-/-}$) exhibit hyperglycemia, impaired glucose tolerance associated with hypoinsulinemia and pancreatic islet abnormalities (malformation, apoptosis and inflammation)[14]. Interestingly, PCSK9, whereas it is not expressed in α- and β-cells, is co-localized specifically with somatostatin in human pancreatic delta-cells, a finding which may be implicated in the previously mentioned results[13]. These findings support the previously mentioned statement concerning the detrimental role of LDLR-associated cholesterol accumulation in pancreatic beta-cells on insulin secretion and carbohydrate homeostasis. Accordingly, three recently published genetic studies showed that PCSK9 variants-associated genetically predicted reduction of LDL-c was related with an increased risk for type 2 diabetes (Table 2)[15-17]. Overall, these observations point to a possible adverse effect of PCSK9 inhibitors on carbohydrate metabolism.

On the other hand, available clinical data have suggested a detrimental association of PCSK9 with glucose metabolism (Table 1). Thus, in children a significant correlation of PCSK9 levels with glucose, insulin, and HOMA-IR levels was observed, while an increase in PCSK9 levels by 1%-2% was associated with 10% higher fasting insulin levels in both sexes[18]. It has been reported that hepatic PCSK9 expression is regulated by insulin *via* the sterol regulatory element-binding protein I-C (SREBP-1C); thus PCSK9 is secreted in an insulin-dependent fashion[19], underlying an association between PCSK9 and carbohydrate metabolism[20]. Additionally, in abdominally obese men PCSK9 levels were associated with dyslipidemia (with small dense LDL particles and increased apolipoprotein CIII levels) but also with insulin resistance (increased HOMA-IR)[21].

The results of the clinical trials, however, do not support any significant effect of these drugs on carbohydrate metabolism (Table 1). In fact, a recently published analysis of 10 phase 3 clinical trials with alirocumab showed that the hazard ratio for diabetes-related treatment adverse effects among 3448 non-diabetic individuals was 0.64 (95%CI: 0.36-1.14) in alirocumab-treated patients *vs* placebo-treated and 0.55 (95%CI: 0.22-1.41) *vs* ezetimibe-treated patients[22]. In prediabetic individuals, the hazard

Table 1 Studies that examined the association of proprotein convertase subtilisin/kexin type 9 with carbohydrate metabolism

Ref.	Type	Main findings
Studies pointing to a positive effect of PCSK9 on carbohydrate metabolism		
Mbikay *et al*[14]	Experimental (mice)	PCSK9-null male mice over 4 mo of age carried more LDLR and less insulin in their pancreas; islets exhibited signs of malformation, apoptosis and inflammation
Awan *et al*[11]	Genetic study	Carriage of the loss-of-function PCSK9 p.R46L mutation was associated with insulin resistance in subjects with apolipoprotein E3/E2 genotype
Studies pointing to a negative effect of PCSK9 on carbohydrate metabolism		
Langhi *et al*[13]	Experimental (mice)	PCSK9 deficiency does not alter insulin secretion and glucose tolerance
Baass *et al*[18]	Clinical study (children)	Significant correlation of PCSK9 levels with glucose, insulin and HOMA-IR levels; an increase in PCSK9 levels by 1%-2% was associated with 10% higher fasting insulin levels in both sexes
Arsenault *et al*[21]	Clinical study (abdominally obese men)	PCSK9 levels are associated with dyslipidemia and with increased HOMA-IR
Studies pointing to a neutral effect of PCSK9 on carbohydrate metabolism		
Bonnefond *et al*[12]	Genetic study	The p.R46L mutation is not associated with markers of glucose homeostasis; p.R46L carriers did not experience an increased risk of new-onset diabetes mellitus
Colhoun *et al*[22]	Analysis of 10 phase 3 clinical trials with alirocumab (3448 non-diabetic individuals)	Hazard ratio for diabetes-related treatment adverse effects 0.64 (95%CI: 0.36-1.14) in alirocumab-treated patients vs placebo-treated and 0.55 (95%CI: 0.22-1.41) *vs* ezetimibe-treated patients
Blom *et al*[23]	Post hoc analysis of the DESCARTES trial (evolocumab)	No changes in parameters of carbohydrate metabolism in patients with pre-existing dysglycemia or metabolic syndrome
Ongoing trials that may better delineate the role of PCSK9 inhibition on carbohydrate metabolism		
Fourier trial (ClinicalTrials.gov Identifier: NCT01764633)	Ongoing trial	Primary hypothesis is that additional LDL-c lowering with evolocumab decreases the risk of cardiovascular events in subjects with clinically evident cardiovascular disease
Odyssey trial (ClinicalTrials.gov Identifier: NCT01663402)	Ongoing trial	Primary hypothesis is that additional LDL-c lowering with alirocumab decreases the risk of cardiovascular events in patients who have experienced an acute coronary syndrome event 4 to 52 wk prior to randomization

PCSK9: Proprotein convertase subtilisin/kexin type 9; LDLR: Low-density lipoprotein receptors; HOMA-IR: Homeostasis model assessment-insulin resistance; LDL-c: Low-density lipoprotein cholesterol.

Table 2 Proprotein convertase subtilisin/kexin type 9 inhibitors and diabetes mellitus: Results of the mendelian randomization studies

PCSK9 variants	Decrease in serum LDL cholesterol	Odds ratio for type 2 diabetes mellitus
rs 11591147[15]	1 mmol/L (38.4 mg/dL)	1.19 (95%CI: 1.02-1.38)
[1]4 independent variants[16] (rs 11583680, rs 11591147, rs 2479109, rs 11206510)	1 mmol/L (38.4 mg/dL)	1.29 (95%CI: 1.11-1.50)
[2]Genetic score[17]	10 mg/dL	1.11 (95%CI: 1.04-1.19)

[1]Associations with fasting glucose, body weight and waist-to-hip ratio were also noticed; [2]The increased risk of diabetes was observed only in individuals with impaired fasting glucose levels. PCSK9: Proprotein convertase subtilisin/kexin type 9; LDL: Low-density lipoprotein; CI: Confidence interval.

ratio associated with transition of prediabetes to new-onset diabetes for alirocumab was 0.90 (95%CI: 0.63-1.29) *vs* placebo and 1.10 (95%CI: 0.57-2.12) *vs* ezetimibe. Furthermore, no change in plasma glucose and glycated hemoglobin (HbA_{1c}) levels was observed between treated groups in non-diabetic individuals of these results[22]. Additionally, a post hoc analysis of the DESCARTES showed that the administration of evolocumab (420 mg monthly) was not associated with any changes in parameters of carbohydrate metabolism in patients with pre-existing dysglycemia or metabolic syndrome[23]. Finally, the available data suggest similar effects of these drugs on the levels of serum lipid parameters in diabetic *vs* non-diabetic individuals[24]. However, the relatively small number of patients, the short-follow up, the design of the studies (administration on top of statin therapy) may reduce the significance of these observations.

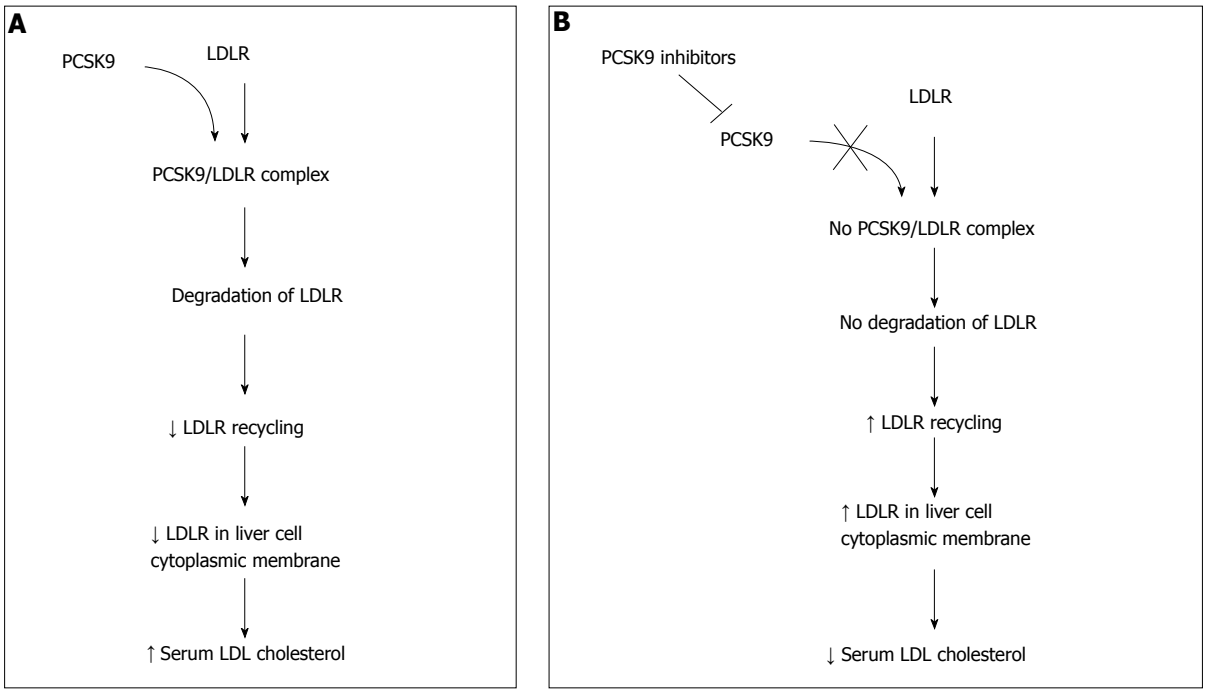

Figure 1 The effect of proprotein convertase subtilisin/kexin type 9 (A) and proprotein convertase subtilisin/kexin type 9 inhibition (B) on liver cells low-density lipoprotein receptors expression and serum low-density lipoprotein-cholesterol levels. PCSK9: Proprotein convertase subtilisin/kexin type 9; LDL: Low-density lipoprotein; LDLR: Low-density lipoprotein receptors.

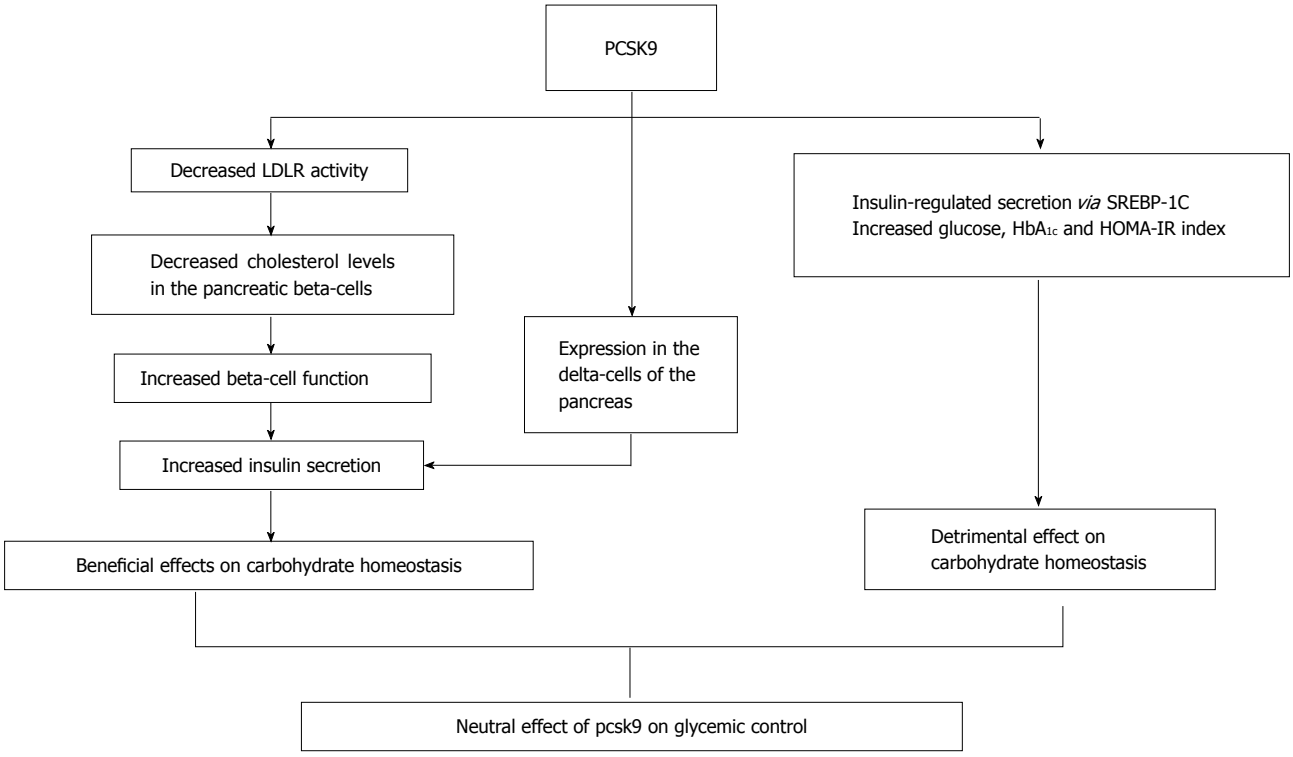

Figure 2 The role of proprotein convertase subtilisin/kexin type 9 on carbohydrate homeostasis. Accordingly, PCSK9 inhibitors may be associated with a neutral effect on carbohydrate homeostasis at least in the short term. PCSK9: Proprotein convertase subtilisin/kexin type 9; LDL: Low-density lipoprotein; LDLR: LDL receptors; HbA1c: Glycated hemoglobin; SREBP-1C: Sterol regulatory element-binding protein I-C; HOMA-IR: Homeostasis model assessment-insulin resistance.

Thus, the effects of PCSK9 and accordingly of PCSK9 inhibitors on carbohydrate metabolism may be seen under different points of view (Figure 2). The potential detrimental consequences of PCSK9 inhibitors on pancreatic cells leading to reduced insulin secretion due to a direct effect on pancreatic cells or to increased intracellular cholesterol levels may be counterbalanced by their direct beneficial effects on carbohydrate

homeostasis. Alternatively, the relatively short duration of the above mentioned clinical trials is not adequate for any detrimental effect of PCSK9 inhibition to be evident. It should be also mentioned that in the clinical trials the addition of PCSK9 inhibitors to statins may have partially masked their effects on glucose metabolism if there are shared mechanisms of action between these two drug classes. Finally, a generally non-significant effect of PCSK9 inhibition on glucose metabolism cannot be excluded. Thus, the results of both Fourier (Clinical Trials.gov Identifier: NCT01764633) and Odyssey (Clinical Trials.gov Identifier: NCT01663402) outcome trials may better delineate the role of PCSK9 inhibitors on the parameters of glucose homeostasis and their long-term effect on the incidence of new-onset diabetes mellitus.

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REVIEW

Obesity, metabolic syndrome and diabetic retinopathy: Beyond hyperglycemia

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Abstract

Diabetic retinopathy (DR) is the most feared ocular manifestation of diabetes. DR is characterized by progressive retinal damage that may eventually result in blindness. Clinically, this blindness is caused by progressive damage to the retinal microvasculature, which leads to ischemia, retinal swelling, and neovascularization. Retinopathy is associated with both type 1 and type 2 diabetes, with DR being the leading cause of new onset blindness in United States adults. Despite this strong association with diabetes, it must be noted that the development of retinopathy lesions is multifactorial and may occur in individuals without an established history of diabetes. Metabolic syndrome is a multifactorial condition of central obesity, hypertriglyceridemia, dyslipidemia, hypertension, fasting hyperglycemia, and insulin resistance. Although several studies examined the individual components observed in the metabolic syndrome in relation to the development of DR, there is conflicting data as to the association of the metabolic syndrome with the development of retinopathy lesions in non-diabetic subjects. This review will summarize the current literature on the evidence of the metabolic syndrome on retinopathy in subjects with and without an established history of diabetes. This review will also discuss some of the mechanisms through which metabolic syndrome can contribute to the development of retinopathy.

Key words: Diabetic retinopathy; Diabetes; Metabolic syndrome; Oxidative stress; Inflammation; Insulin resistance

Core tip: Metabolic syndrome is a multifactorial condition

of central obesity, hypertriglyceridemia, dyslipidemia, hypertension, and fasting hyperglycemia and insulin resistance. Although several studies examined the individual components of the metabolic syndrome in relation to development of diabetic retinopathy, there is conflicting data as to the association of the metabolic syndrome with the development of retinopathy lesions in non-diabetic subjects. This review examined the current literature on the prevalence and impact of the components of the metabolic syndrome on the development of retinopathy in subjects with and without an established history of diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is a pathologic condition affecting approximately 29 million or 9% of people in the United States[1]. DM is a condition classified by metabolic disorder caused by chronic hyperglycemia that results in a number of pathologies including microvascular and macrovascular complications such as retinopathy, neuropathy, nephropathy, ischemic heart disease, cerebrovascular disease and peripheral vascular diseases[2,3]. Classically, DM has two etiologies that are classified as either type 1 or type 2. The hyperglycemia in type 1 DM is a direct result of destruction of the pancreatic beta cells; whereas, the hyperglycemia seen in type 2 DM is a result of insulin resistance and subsequent pancreatic beta cell dysfunction[4-6].

SECONDARY DIABETES

DM may occur due to other endocrine disorders such as pituitary, adrenal and/or thyroid diseases (reviewed in[7]). These endocrine disorders are associated with sustained release of hormones that are antagonistic to insulin action including growth hormone, glucocorticoids, catecholamines or glucagon. Therefore, patients with acromegaly, Cushing syndrome, pheochromocytoma, primary hyperaldosteronism, hyperthyroidism, or glucagonoma are at high risk to develop secondary diabetes[8,9]. Chronic alcoholic pancreatitis can also result in secondary DM, where diabetes develops mainly due to deficiency in insulin[10]. A recent study identified incidence of slight to moderate diabetic retinopathy in 12.5% patients with acromegaly[11]. Nevertheless, there are no large or comprehensive studies on development of microvascular complication in general or diabetic retinopathy in particular in patients with secondary-DM.

Pre-diabetic state "the metabolic syndrome"

Although the ADA guidelines for a clinical diagnosis of type 2 diabetes require a random blood sugar greater than 200 mg/dL or fasting blood sugar greater than 125 mg/dL, current evidence suggests that the body undergoes significant metabolic changes prior to the development of frank diabetes[1,12]. These changes include the following: Insulin resistance and the associated hyperinsulinemia and hyperglycemia, vascular endothelial dysfunction partially due to inappropriate release of cytokines from adipose tissue[13-15]. Clinically, the metabolic syndrome has several definitions but is generally diagnosed in individuals presenting with 3 of the criteria listed on Table 1: Central obesity, hypertriglyceridemia, dyslipidemia, hypertension, and fasting hyperglycemia[15-17]. Current population studies have found that the metabolic syndrome affects a large number of individuals. One study in particular found its prevalence to be roughly 22% of adults in the United States, more significantly, an age-dependent increase in prevalence was also found[18]. Furthermore, a more recent study found that prevalence increased to roughly 34.5%[19]. Of note, the presence of the metabolic syndrome increases an individual's risk of developing type 2 diabetes, cardiovascular disease, and all-cause mortality from cardiovascular disease[20-22].

DEVELOPMENT OF RETINOPATHY IN SUBJECTS WITHOUT A HISTORY OF DIABETES

Retinopathy has been defined in different studies to include microaneurysms, retinal hemorrhages, hard exudates, cotton wool spots, retinal venular abnormalities (venous beading and tortuosity), intraretinal microvascular abnormalities, and new blood vessels[23,24]. Although, hyperglycemia and hypertension are strongly associated with incident retinopathy, there are other etiologies including ocular and systemic causes. Ocular etiologies include central or branch retinal vein occlusion, retinal telangiectasia ("spider veins"), and retinal macroaneurysms[24]. Systemic causes range from the hypertension, carotid atherosclerotic disease, previous head radiotherapy, severe forms of all anemias, and other blood abnormalities such as sickle cell. Systemic diseases such as lupus, toxoplasmosis, and acquired immune deficiency syndrome have also been associated with the development of retinopathy lesions in patients with no history of diabetes[24,25]. There are several studies that examined the association of the components of metabolic syndrome with the development of retinopathy lesions in non-diabetic subjects. With this in mind, the focus of this review will primarily be the impact of metabolic syndrome on the development of retinopathy lesions in patients with established history of primary-DM or without history of diabetes. This review will also discuss some of the mechanisms through which metabolic syndrome can contribute to the development of retinopathy.

Table 1 Adult treatment panel III criteria for diagnosis of the metabolic syndrome[1]

⩾ 3 of the following	
Fasting glucose	⩾ 6.1 mmol/L (110 mg/dL)
HDL cholesterol	Male: < 1.0 mmol/L (40 mg/dL)
	Female: < 1.3 mmol/L (50 mg/dL)
Triglycerides	⩾ 1.7 mmol/L (150 mg/dL)
Abdominal obesity	Male waist circumference: ⩾ 102 cm
	Female waist circumference: ⩾ 88 cm
Blood pressure	⩾ 130/85 mmHg

[1]http://www.nhlbi.nih.gov/files/docs/guidelines/atglance.pdf (acquired 2016 Jul 17[136]).

DIABETIC RETINOPATHY

Among the microvascular complications of diabetes, diabetic retinopathy (DR) is among the most feared one. Retinopathy has traditionally been viewed as a product of ischemic insult; however, this topic is well documented in other reviews[26,27]. DR is broadly classified into two stages: Nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). Classification is determined by the presence of neovascularization in the retina[27]. NPDR typically precedes PDR and is divided into the following stages: Mild, moderate, severe, and very severe. These stages are based on the likelihood that the retinopathy will progress to PDR. Clinically, a patient with NPDR presents with microvascular abnormalities such as microaneurysms and hemorrhage, affecting the macula and posterior retina. Vascular abnormalities, such as an increased permeability of the retinal vasculature and serum leakage, contribute to capillary loss and subsequent ischemia[27]. PDR is defined by the presence of neovascularization and is divided into the following stages: Early, high risk, and severe neovascularization[27]. In response to retinal hypoperfusion, an increase in local production of vasoproliferative factors such as vascular endothelial growth factor (VEGF)[27] and platelet-derived growth factor (PDGF)[28,29] occurs as a maladaptive protection mechanism. Increased levels of VEGF are traditionally correlated with stabilization of the transcription factor hypoxia-inducible factor-1 (HIF-1) levels under hypoxic conditions[30]. Both VEGF and PDGF are strongly associated with neovascularization *via* induction of new vascular development typically from optic disc or retinal vessels[31,32]. This neovascularization further compounds the damage by contributing to the development of preretinal and vitreous hemorrhage, fibrosis, potential retinal detachment, and blindness[33-35].

CURRENT THERAPEUTICS FOR DR

The mainstay standard of care for DR is the laser treatment, a highly effective procedure to slowdown visual loss in patients with PDR. The laser-mediated photocoagulation seals leaking blood vessels directly or by eliminating abnormal newly formed blood vessels in the periphery of the retina that is thought to be involved in VEGF production[36]. With VEGF being a common product strongly associated with the progression of DR, current pharmacologic treatment strategies have been based on its local inhibition within the retina[37]. Anti-angiogenic therapy was developed in attempt to improve vision in patients with diabetic macular edema (DME) as well as PDR. Indeed, monthly injections of ranibizumab, an anti-VEGF improved vision, reduced the risk of further vision loss. These results were observed after 2-years and were sustained for 3-years[38]. Anti-VEGF treatment improved macular edema in diabetic patients as well as when it was used in combination with panretinal photocoagulation in patients with PDR. The reported side effects of ranibizumab in "as - needed" treatment regimen over a 5-year.

INSULIN RESISTANCE AND THE METABOLIC SYNDROME

Researchers have proposed several mechanisms for the development of insulin resistance and the metabolic syndrome. These include: Genetic defects in proteins involved in the insulin action cascade, increased levels of visceral adiposity, free fatty acid levels (FFA), and chronic inflammation[39,40]. Insulin resistance in adipose tissue, regardless its molecular or environmental basis, causes decrease in FFA uptake by fat cells and/ or increase in FFA release from fat cells. Under the insulin resistant state, there is impaired glucose handling by skeletal muscle and adipose tissue. This impaired glucose intake is a significant contributor to the hyperglycemia and associated vascular endothelial damage observed in insulin resistant individuals[41,42]. Additionally, insulin is important in the signaling for nitric oxide release from vascular endothelial cells, resulting in vasodilation and reduced vascular resistance, which reduces blood pressure[43,44]. Thus, there is a strong associations between the presence and extent of insulin resistance with hypertension due to increased vascular resistance and impaired glucose regulation[45,46].

Hypertension, affecting 29.8% of United States adults[47] represents the best known systemic condition associated with non-diabetic retinopathy. Hypertension is an established risk factor for the development of several cardiovascular complications including retinopathy, atherosclerosis, and aneurysms[48,49]. Poorly controlled systemic hypertension causes worsening of microvascular disease of the eye like DR[50]. Hypertensive retinopathy shared the pathophysiology of damaged retinal vascular endothelium similar to DR[51,52]. In contrast to the metabolic damage in DR, this vascular endothelial damage is mechanically induced by increased blood flow[53,54]. Despite the relationship between retinopathy and hypertension in patients without history of diabetes, one study, "the Hoorn", identified retinopathy 8 of the 17 individuals without history of diabetes who developed retinopathy did not

have hypertension. In addition, HbA1c level and waste to hip ratio (WHR) were risk factors in the nondiabetic individuals[23]. These finding suggest that retinal pathologies begin to develop prior to a clinical diagnosis of hypertension that eventually result in retinopathy.

RETINOPATHY IN SUBJECTS WITH METABOLIC SYNDROME BUT NO HISTORY OF DIABETES

In the absence of a clinical diagnosis of diabetes, associations have already been found between the metabolic syndrome and macro- or microvascular pathologies such as atherosclerosis, arteriosclerosis, and endothelial dysfunction[55,56]. Several studies examined the associations between the independent components of the metabolic syndrome with the development of retinal vascular injury, by measuring the mean retinal artery and venous caliber[31]. In this study, components of the metabolic syndrome including large waist circumference, lower HDL cholesterol levels, and higher BP were independently associated with reduced mean retinal arterial caliber in non-diabetic persons. Individuals with hypertriglyceridemia were significantly more likely to have arteriovenous nicking and later develop retinopathy[31]. These finding clearly show an association between the MS and retinal vascular dysfunction.

Following the earlier notion that dyslipidemia plays a critical role in DR, several studies examined the individual components observed in the metabolic syndrome in relation to DR[57-60]. Similar to the impact of dyslipidemia, there is conflicting data as to the association of the metabolic syndrome with the development of retinopathy lesions in non-diabetic subjects. One study of obese individuals older than the age of 40, found no significant correlation between the metabolic syndrome and retinopathy once diabetes and hypertension were controlled for[61]. Another population study found no significant association in the incidence of retinopathy and the metabolic syndrome in the non-diabetic population, but there was a significant association between hypertension and retinopathy[57].

In contrast, studies focusing on specific patient populations found differing results. A recent study in a Chinese population identified a positive correlation between the metabolic syndrome and retinopathy in the examined non-diabetic subjects[62,63]. In a study of Japanese adults, the metabolic syndrome was found to be associated with retinopathy; a larger waist circumference was associated with wider venular diameter and retinopathy lesions; a higher blood pressure level was associated with focal arteriolar narrowing, arteriovenous nicking, enhanced arteriolar wall reflex and narrower arteriolar diameter; and a higher triglyceride level was associated with enhanced arteriolar wall reflex[64]. In the Hoorn study, in the Netherlands, there was significant correlation of retinopathy with the combination of high waste-to-hip ratio (WHR), HbA1c level, and hypertension in non-diabetics and in glucose-impaired subjects, supporting a role for insulin resistance in the pathogenesis of retinopathy[23].

Interestingly, there was no significant correlation between incidence of retinopathy with serum levels of triglycerides, and total cholesterol or body mass index (BMI)[23]. Despite generalized obesity indicated by high BMI not being associated with retinopathy, a high-body-fat percentage indicated by WHR has been shown to be significantly associated with development of retinopathy in patients with type-2 diabetes[65]. Similar to the Hoorn study, the WHR was also an independent risk factor in the diabetic patients in the EURODIAB study[66]. These discrepancies speak to a number of possible factors including the inability of the BMI calculation to accurately estimate body composition[67,68] while the WHR is an indicator for central obesity and is associated with insulin resistance[69]. In addition, differences in measurement methods and quantification for incidence and/or rate of progression of retinopathy[57,70,71]. The other factor is that dysfunction of adipose tissue has been shown to increase oxidative stress and subsequent cytokine, contributing to the pathogenesis of retinopathy[72]. Given that hyperglycemia and hypertension are the strongest risk factors for the development of retinopathy lesions, and that these two conditions are contributors to the diagnosis of the metabolic syndrome, it may be beneficial to modify the clinical approach to individuals with the metabolic syndrome, namely those with hypertension and hyperglycemia coupled with obesity, calculated by WHR, in order to prevent or slow the development of hyperglycemia, hypertension, and hypertriglyceridemia, which in turn could possibly delay the onset of retinopathy lesions and visual impairment in subjects with these comorbidities.

RETINOPATHY IN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

Although insulin resistance is a key pathogenic factor in both non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome, few studies examined the relationship between NAFLD and retinopathy in the presence or absence of diabetes. Central adiposity and visceral fat are important source of triglycerides leading to steatosis and NAFLD[73]. The prevalence increases in subjects with impaired glucose tolerance (43%) and in subjects with newly diagnosed DM[74]. The NHANES Ⅲ was conducted by the Centers for Disease Control and Prevention using a nationwide probability sample of the United States non-institutionalized civilian population from 1988 to 1994. While a strong association between diabetes and retinopathy was observed, NAFLD was not associated

with retinopathy in the non-diabetic population[75]. No significant relationship between NAFLD and incident retinopathy was observed in either diabetic or non-diabetic after adjusting for the confounders such as age, gender, ethnicity, and metabolic components. In addition, this same study found no significant increase in DR prevalence in individuals with both DM and NAFLD[75]. In contrast, prior studies observed a positive association between pediatric NAFLD participants and the degree of retinopathy signs[76]. Additionally, NAFLD was associated with increased rates of chronic kidney disease and proliferative diabetic retinopathy in individuals with type 2 diabetes in Italy[77]. Of note, NAFLD was not significantly correlated with the incidence of retinopathy in patients with NPDR after adjusting for multiple factors[77].

RETINOPATHY IN PATIENTS WITH METABOLIC SYNDROME AND HISTORY OF DIABETES

Traditionally, the development of DR in patients with type 1 or type 2 diabetics has been linked to the associated hyperglycemia[78]. Whether the existence of metabolic syndrome in these patients can accelerate or aggravate the incidence of DR is not clear. For example, the findings of the landmark studies, Land mark clinical trials including United Kingdom Prospective Diabetes Study (UKPDS) in patients with type-2 diabetes[79,80], the Diabetic Control and Complications Trial (DCCT), in patients with type-1 diabetes[81] and its follow-up, the Epidemiology of Diabetes and Interventions and Complications (EDIC)[82,83] were traditionally interpreted that tight glycemic control significantly delayed development of DR. However, the level of reduction was significantly lower in patients with type 2 diabetes (25%) and 76% in patients with type 1 diabetes, suggesting that factors outside of hyperglycemia associated with type 2 diabetes may play a role in the pathology of the microvascular complications such as retinopathy[82,83]. Also, the tight metabolic control, individually and coupled with other interventions, has been shown to significantly decrease the incidence of retinopathy, while also increasing the quality and duration of life in these patients[82]. With this population beginning to live longer, the rates and incidence of comorbid metabolic syndrome and type 1 diabetes has begun to increase as this population begins to be more representative of the general United States population[84].

Another study in patients with type 1 diabetes found that tight glycemic control had threshold effectiveness at reducing the incidence of retinopathy. When looking for other associations, they found that once the duration of hyperglycemia was controlled for, increased WHR and fasting triglyceride levels were the only other factors strongly associated with the incidence of retinopathy in these patients[85]. Interestingly, a study in Belgium found that patients with type 1 diabetes who are overweight and had higher BMI had more retinopathy than normal-weight diabetic patients. Patients with retinopathy were older and had a longer diabetes duration and higher A1C than individuals without retinopathy[86]. However, one study took a different approach to studying this relationship by estimating the prevalence of DR in individuals with the metabolic syndrome depending on the number of MS components these individuals had[65]. This study focused on diabetic patients but normalized for several associated parameters including HbA1C. This study found a linear relationship between the number of MS components and the prevalence of DR[65]. These findings support the relationship between the metabolic syndrome, namely the obesity and hypertriglyceridemia, and the development and/or progression of DR. Given that these conditions are strongly associated with type 2 diabetes, and are components of the metabolic syndrome, it would be logical to look into their contribution to the incidence of retinopathy in this population[87]. Other studies found positive correlations between the comorbid metabolic syndrome and type 2 diabetes with all cardiovascular complications including DR[88,89]. Furthermore, other studies found that the presence of hyperinsulinemia and dyslipidemia in type 2 diabetics was associated with the onset of microvascular complications[90,91]. A case-controlled study, with data obtained from 2551 Chinese participants found that the trend to develop DR with metabolic syndrome was significantly higher than that without metabolic syndrome. Metabolic syndrome was an independent statistical indicator of the presence of DR after adjusting for age and sex as well as HbA1c and duration of diabetes[65]. Additively these findings bolster the claim that in addition to hyperglycemia and hypertension, the hypertriglyceridemia seen in several individuals in this population may very well play a significant role in the pathogenesis of DR in this population.

DYSLIPIDEMIA AND DEVELOPMENT OF DR

Diabetic dyslipidemia is traditionally characterized by high plasma triglycerides and low-density lipoprotein cholesterol (LDL) and reduced levels of high density lipoprotein cholesterol (HDL). While early studies have suggested positive correlation between dyslipidemia and hard exudates, longitudinal studies in patients with type-1 diabetes found modest impact of increased total cholesterol and HDL on the incidence of DR[92]. Of note, serum triglyceride levels were not considered in that study. As discussed in the review by Sabanayagam *et al*[93] 2016, changes in the circulating levels of lipids are not always associated with DR progression. A recent meta-analysis revealed that the triglycerides, total cholesterol and LDL cholesterol were significantly

elevated in persons with diabetic macular edema (DME) when compared to those with DM without DME[94]. In the Madrid Diabetes Study, higher LDL cholesterol level increased the 4-year risk for DR by 8-fold in type 2 diabetes[95]. In contrast, higher levels of total and LDL cholesterol were found to be protective of any retinopathy in a Singapore study as well as in a multi-ethnic United States population study[96,97]. In a follow-up analysis of the DCCT-EDIC from type-1 diabetic patients, the severity of retinopathy was positively associated with triglycerides (combined cohort) and negatively associated with HDL cholesterol in men from combined cohort[98]. Advanced lipoprotein profiling identified positive association of retinopathy with small and medium VLDL and negatively with VLDL sizes. No associations were found with apolipoprotein-A1, Lipoprotein(a), or susceptibility of LDL to oxidation in type-1 diabetic patients.

The early belief that dyslipidemia plays a critical role in DR, initiated several studies that examined the impact of lipid lowering drugs on DR. As discussed in the review by Modjtahedi *et al*[99] 2016, overall there are variable results but the majority of the studies support a protective role of some lipid lowering agents such as fenofibrate in mitigating hard exudates and DR. The beneficial effects of lipid lowering agents are not fully attributed to correcting dyslipidemia and may be attributed to anti-inflammatory, antioxidants and anti-apoptotic effects. Finally, two major randomized clinical trials showed that fenofibrate, a drug that is used to reduce cholesterol significantly inhibited DR progression in diabetic patients[100,101]. Lastly, a multinational case-control study suggested that conventional serum lipids profiles are unlikely to show clear and dependent effects on the development of DR[102]. The published literature suggested an association between diabetic dyslipidemia and DR; however, a more detailed and specific subtype of lipids or lipoproteins may have a clear pathogenic role rather than traditional lipid profile. In addition, alternative hypothesis suggests that initial damage to the retina barrier facilitate leakage of lipids and its oxidized metabolite to exert local adverse effects that are not necessarily mirrored by significant alteration in serum lipid profile[99].

MECHANISMS ASSOCIATED WITH RETINOPATHY IN THE METABOLIC SYNDROME

DR is classically perceived as microvascular disease with initial vascular endothelial damage as a direct result of hyperglycemia. Given the known pathophysiology of the components of the metabolic syndrome, as well as its association with type 2 diabetes[46,103], we will discuss the common major mechanisms of pathology in both the metabolic syndrome and diabetes.

For retinopathy in patients with an established history of diabetes, hyperglycemia has been identified as primary factor evident by the strong correlation between an individual's HbA1c and the development of DR[79]. Results from the clinical trial UKPDS in patients with type 2 diabetes[79,80] and the DCCT in patients with type 1 diabetes[81] established that intensive glycemic control significantly reduced the incidence of retinopathy. More specifically, risk reduction of DR was found to be 76% in patients with type 1 diabetes and 25% in type 2 diabetics. Several studies examined mechanisms involved in hyperglycemic damage include non-enzymatic glycosylation of vascular basement membrane, advanced glycation end products and osmotic damage due to the conversion of circulating sugars to sorbitol by aldose reductase[78,104,105]. Studies have found that diabetes mellitus and the metabolic syndrome both, increase reactive oxygen species (ROS) production and decrease the antioxidant capacity. This is associated with oxidative damage of cell components such as proteins, lipids, and nucleic acids can trigger a chronic inflammatory response[106,107]. Impact and sources of hyperglycemia-derived oxidative stress and proinflammatory cytokines in the diabetic retina are well-documented in the literature[108,109]. As depicted in Figure 1, the aforementioned mechanisms result in vascular endothelial cell dysfunction and an increase in local immune cell activity resulting in a leukocyte oxidative burst and the associated increased leukostasis, vascular permeability[110,111]. Inflammation-mediated leukostasis has been linked to pericyte and endothelial cell death, retinal ischemia, and neovascularization, which contribute to vision loss in DR[109].

In contrast, identifying mechanisms involved in retinopathy associated with the metabolic syndrome is not a straightforward task. The metabolic syndrome is a combination of several criteria (Table 1) including central obesity, hypertriglyceridemia, insulin resistance, dyslipidemia, hypertension, and fasting hyperglycemia. Furthermore, central obesity and the excess adipose tissue observed in obese individuals partially contribute to the development of the insulin resistance syndrome and cardiovascular disease. As depicted in Figure 2, metabolic syndrome and endocrine dysfunction of adipose tissue can be very important in the development of retinopathy lesions and progression of the DR.

ADIPOSE TISSUE DYSFUNCTION AND ADIPOKINES

In healthy adults, adipose tissue secretes a number of factors such as resistin and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin-6 (IL-6), which participate in the activation of macrophages that further perpetuate this inflammatory cascade. When adipose tissue is accumulated, as it is in obesity due to increased adipose tissue, it begins to operate in a dysfunctional manner. Additionally, in the

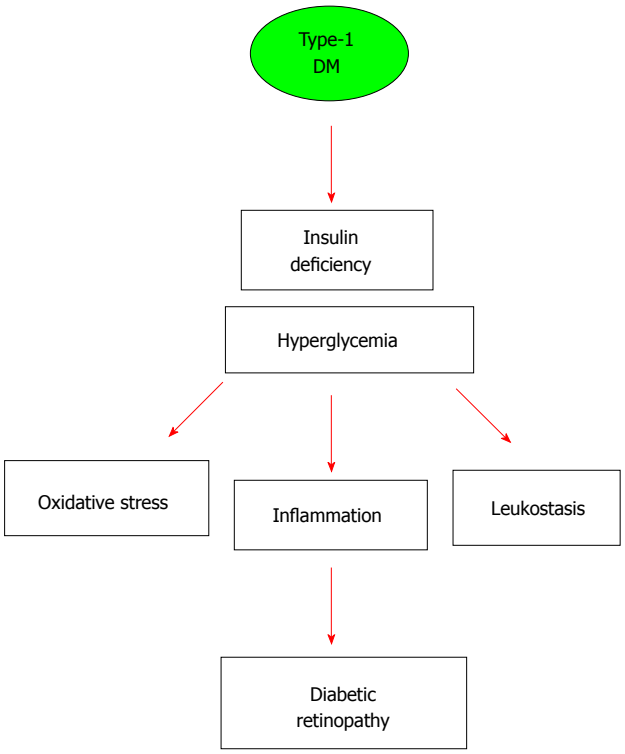

Figure 1 Schematic presentation of the postulated primary role of hyperglycemia and insulin deficiency in driving diabetic microvascular complications in type-1 diabetes. Secondary mediators of retinal vascular damage include oxidative stress, inflammation and leukostasis.

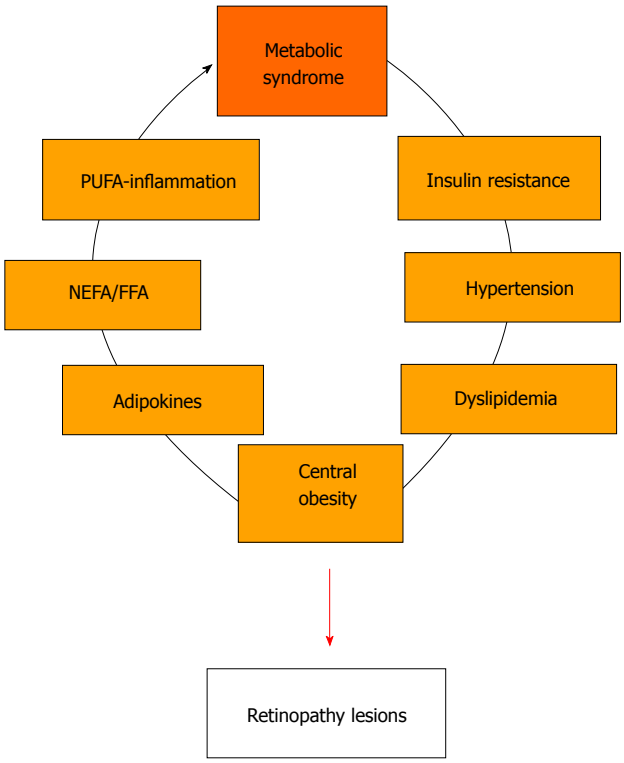

Figure 2 Schematic presentation of the multiple pathways involved in metabolic syndrome including hypertension, central obesity, dyslipidemia, adipose tissue and adipokine's dysfunction, altered free fatty acids levels and polyunsaturated fatty acid metabolism leading to local and systemic inflammation. FFA: Free fatty acids; PUFA: Polyunsaturated fatty acid.

obese state, adipose tissue secretes reduced amounts of the anti-inflammatory adipokine adiponectin, an adipokine that normally acts to increase insulin sensitivity by inhibiting hepatic gluconeogenesis and augmenting skeletal muscle glucose uptake[14,112]. In addition to these factors, it appears that excess adipose tissue leads to dysfunctional release of several other factors including retinol binding protein-1 and leptin[9,14]. The abnormal release of these factors contribute to insulin resistance by further dysregulating glucose homeostasis and augmenting hyperphagia and hyperglycemia induced damage[14].

Recently, the endocrine function of adipose tissue has become the focus of several studies. These functions include the secretion of adiponectin and resistin, both of which are involved in insulin sensitization, and subsequent increased glucose uptake[14,113]. Adiponectin functions to increase insulin sensitivity and is secreted at high levels in lean individuals with low levels of adipose tissue. However, as adipose tissue increases, the level of adiponectin secreted by adipose tissue decreases partially due to the increased secretion of pro-inflammatory markers and their associated induction of oxidative stress[14,113,114]. Another significant adipokine, resistin, is abnormally increased in obese individuals and is a contributor to the development and progression of the insulin resistance[14,115,116]. Dysfunctions in these processes, independent of causing direct damage to vascular endothelium, can contribute to insulin resistance and hyperglycemia. One study looking at the progression from non-proliferative to proliferative, found a strong association between the progression of retinopathy and increased c-reactive protein levels and increased serum resistin, a protein secreted by several tissues including white adipose tissue, nonfat cells of adipose tissue, bone marrow, and lung. This study found that increased serum resistin was associated with obesity and was highest in obese individuals with diabetes. Furthermore, increased serum resistin was associated with increased triglycerides and the progression of retinopathy[117,118]. These findings further bolster the association between DR and hyper-triglyceridemia particularly due to resistin's association with obesity and increased triglycerides. Furthermore, resistin's association with retinopathy is made more plausible given its association with type 2 DM, insulin resistance, and inflammation. All of these factors have demonstrated associations with DR; however, the specific role of resistin in inflammation has not yet been elucidated[117,119]. Given that c-reactive protein is a known inflammatory biomarker related to vascular endothelial dysfunction and atherogenesis, its association with increased serum resistin levels provides additional support for adipose tissue dysfunction in the obese state contributing to the pathologies associated with diabetes including DR[117,118].

ADIPOSE TISSUE AND FFA

Adipose tissue can contribute to insulin resistance by regulating the level of the FFAs or the non-esterified fatty acids (NEFAs) depending on the body's energy status[120,121]. Circulating NEFAs/FFAs reduce adipocyte and muscle glucose uptake, and also promote hepatic glucose output. Because lipolysis in adipocytes is depressed by insulin, insulin resistance from any cause can lead to NEFA elevation, which, in turn, induces additional insulin resistance as part of a vicious cycle that eventually contribute to diabetic complication including DR[120,121]. Furthermore, a prior study found that a long-acting antilipolytic drug could lower FFA levels in 9-lean control subjects, 13-obese nondiabetic subjects, 10-obese subjects with impaired glucose tolerance, and 11 patients with type 2 diabetes[122]. The results showed FFA lowering drugs improved oral glucose tolerance in both lean and obese nondiabetic subjects and in obese patients with type 2 diabetes, supporting the notion that adipose tissue can contribute to the pathologies associated with insulin resistance[122].

INFLAMMATION

The involvement of the inflammatory pathway in retinopathy associated with metabolic syndrome is critical in the light of the observation that obesity, hypertension, hyperlipidemias, and insulin resistance are considered low-grade systemic inflammatory conditions[123]. A major contributor to this inflammation is the release of pro-inflammatory cytokines by immune cells such as macrophages, lymphocytes, and leukocytes after they infiltrate the adipose tissue[14,124,125]. The pro-inflammatory cytokines secreted by adipose tissue include interleukins (ILs), notably IL-1β, IL-6, and tumor necrosis factor alpha[126]. These cytokines contribute to the impairment of glucose homeostasis, insulin signaling and development of insulin resistance and cardiovascular complications such as DR[127-130].

The retina is rich in n-3 polyunsaturated fatty acids (PUFAs) and upon activation of phospholipase A2, release of the metabolites arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in response to various stimuli including growth factors, cytokines and free radicals[131]. The AA, EPA and DHA are metabolized by cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP450) enzymes[123]. The AA forms a precursor to pro-inflammatory prostaglandins, thromboxanes and leukotrienes in general. It is noteworthy that AA can also give rise to lipoxins, which are potent anti-inflammatory molecules. Similarly, EPA gives rise to resolvins and DHA to protectins, which possess significant anti-inflammatory properties. As such, these molecules may have a role in modulating the chronic inflammatory state observed in DR and metabolic syndrome[131].

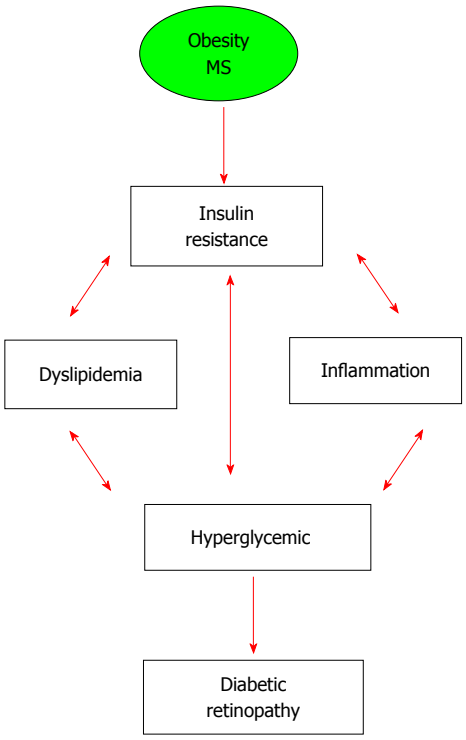

Figure 3 Schematic presentation of the postulated multifactorial and interrelated pathways of insulin resistance, inflammation, dyslipidemia and hyperglycemia that collectively drive microvascular complications in patients with metabolic syndrome with and without established history of diabetes.

Inflammation in DR has also been linked to induction of the cyclooxygenase-2 (COX-2) pathway. Induction of the COX-2 pathway results in a dysfunctional increase in the production of prostaglandins and thromboxane[132]. This dysfunctional production contributes to the chronic inflammatory pathologies by augmenting the vascular permeability noted in these patients, increasing platelet aggregation and leukostasis, which results in the increased local production of pro-inflammatory cytokines[32]. Furthermore, the increased products of the COX-2 pathway in the diabetic patients, has been associated with the increased local secretion of VEGF in the retina, a major contributor to the progression of DR[133,134]. Prior studies have shown that PUFAs, especially EPA and DHA, inhibit the production of both IL-6 and TNF-α and suppress the expression of VEGF[135]. Leukotrienes have been found to be increased in the retinas of diabetic mice. These inflammatory factors contribute to several of the dysfunctions noted chronic retinal inflammation including the increased vascular permeability and increased production of free radicals[131].

CONCLUSION

The prevalence of DM and its associated pathologies have significantly increased as a result of increasing prevalence of obesity and insulin resistance. For retinopathy in patients with established history of DM, hyperglycemia remains the most consistent risk

factor for DR in type 1 diabetes across different studies and populations (Figure 1). While blood pressure is an important risk factor for DR in type 2 diabetes, correlation with serum levels of lipids are not consistent. Meanwhile, considerable evidence showed that central obesity, insulin resistance and dyslipidemia are associated with retinopathy lesion in non-diabetic and diabetic patients. As depicted in Figure 3, the cascade of events involved in development of DR is complex and interrelated and not entirely driven by hyperglycemia in patients with metabolic syndrome. Moreover, diabetic macular edema rather than PDR is the increasingly common cause of visual impairment. Patients with type 2 diabetes with dyslipidemia show higher tendency for DME and can benefit from cholesterol reducing agents such as fenfibrate in addition to antidiabetic agents. Although the current anti-VEGF therapies are beneficial in some patients with DR, about 50% of other patients do not respond adequately. This calls for additional studies to find better treatment and management strategies to prevent its currently incurable complications, such as DR, should be of high priority. Therefore, targeting components of the metabolic syndrome could be a beneficial preventative/dilatory intervention. Specifically, understanding the pathways involved in dysfunction of adipose tissue and the associated alteration of PUFA, FFA and adipokines such as adiponectin and resistin contribute to inflammation and cardiovascular complications including retinopathy lesions in both diabetics and non-diabetics. Novel strategies to suppress systemic and local inflammation seen in DR should be further explored in order to prevent and/or delay DR.

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MINIREVIEWS

PTPN22 and islet-specific autoimmunity: What have the mouse models taught us?

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Abstract

An allelic variant of the protein tyrosin phosphatase non-receptor 22 (*PTPN22*) gene, PTPN22 R620W, constitutes the strongest non-HLA genetic risk factor for the development of type 1 diabetes (T1D). A number studies using mouse models have addressed how PTPN22 predisposes to T1D. PTPN22 downmodulation, overexpression or expression of the variant gene in genetically manipulated mice has generated controversial results. These discrepancies probably derive from the fact that PTPN22 has differential effects on innate and adaptive immune responses. Moreover, the effects of PTPN22 are dependent on other genetic variables. Here we discuss these findings and try to explain the discrepancies. Exploring the mechanism by which PTPN22 contributes to islet-specific autoimmunity could help us understand its role in T1D pathogenesis and exploit it as a potential therapeutic target to prevent the disease.

Key words: Protein tyrosin phosphatase non-receptor 22; Type 1 diabetes; Genetic susceptibility; Mouse model; Autoimmunity; Islet-specific autoimmunity

Core tip: Protein tyrosin phosphatase non-receptor 22 (*PTPN22*) is the strongest non-HLA gene associated with type 1 diabetes (T1D) and many other autoimmune diseases. Several studies using mouse models have generated controversial results on how PTPN22 predisposes to T1D. In our manuscript we summarize these results and try to explain the discrepancies. Our analysis reveals that PTPN22 assumes different roles in innate and adaptive immunity and its effect is strongly dependent on other genetic variables. Hence, additional studies are required to better understand the mechanism by which PTPN22 predisposes to T1D and to exploit it as a potential therapeutic target in T1D and other autoimmune diseases.

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INTRODUCTION

Autoimmune diseases are a type of disorders characterized by abnormal immune responses against self tissues and organs that are subjected to continuous inflammation leading to their demise. Both genetic predisposition and environmental factors are implicated in autoimmune disease pathogenesis[1]. Great amount of research has led to the identification of several disease susceptibility genes; however, the immunological malfunctions that these genes introduce are often poorly understood. In this review we focus on the autoimmune predisposing gene protein tyrosin phosphatase non-receptor 22 (*PTPN22*), which is associated with multiple autoimmune diseases and among them type 1 diabetes (T1D)[2-6]. *PTPN22* encodes a protein tyrosine phosphatase, which plays key roles in innate and adaptive immunity. PTPN22 is involved in T cell receptor (TCR), B cell receptor (BCR) and innate immune signaling and controls the threshold of immune activation and consequently the outcome of an immune response[7,8]. Here, we discuss recent research on the role of PTPN22 in islet-specific autoimmunity experimentally addressed by mouse models. Our aim is to summarize the current knowledge on PTPN22, as raising from mouse model studies, and to highlight the unmet research needs on its role in autoimmunity.

T1D

T1D is an autoimmune disease mediated by autoreactive $CD4^+$ and $CD8^+$ T cells that infiltrate the pancreas and destroy insulin-secreting beta cells[9]. Beta-cell loss results in the reduced production of insulin, which is essential for the glucose metabolism. This condition is life-threatening unless patients undergo substitution therapy, which is based on self-administration of insulin for the rest of their lives. However, insulin replacement is not a cure and despite tight glycemic control, a number of secondary complications can emerge such as heart and kidney disease[9,10]. T1D is a multifactorial disease where genetic and environmental factors contribute to the loss of immunological tolerance to beta-cell antigens[11-13]. Several years elapse between the initial stages of the autoreactive response and the onset of clinical diabetes. During this preclinical phase, autoantibodies (AAbs) against beta-cell antigens emerge, which are currently the most reliable predictive biomarker of the disease. The presence of multiple islet-specific AAbs together with metabolic parameters and particularly dysglycemia can predict with approximately 90% accuracy the development of T1D[14,15]. However, we still lack biomarkers that will reliably indicate the dynamic loss beta cells and predict the emergence of the disease[16].

Islet-specific T cells play central role in the pathogenesis of T1D. They kill beta cells and promote the development of AAbs through their B-cell helper activity[17,18]. As such, they have become the focus of extensive research with the aim to be used as targets for immunotherapy and as biomarkers in prediction and therapeutic studies[19-21]. Important but less recognized is the role of B cells and AAbs in the autoimmune process. Autoreactive B cells are thought to play key role in the development of islet-specific autoimmunity by promoting the presentation of beta-cell antigens to autoreactive T cells, which in turn by providing B-cell help signals, promote the production of AAbs[22]. This autoreactive process has been postulated to take place predominantly within germinal centers (GCs), specialized structures in secondary lymphoid organs where the maturation of B cells into long-lived plasma cells and memory B cells takes place[23,24].

T follicular helper (TFH) cells, a subset of CD4 T cells, are essential for the formation of GCs and the development of optimal antibody responses by guaranteeing the survival of B cells presenting high affinity antigens[25]. The exact mechanism by which autoreactive B cells are eliminated during the GC response is not fully understood, but TFH cells and a subset of FOXP3 regulatory T cells [follicular regulatory T (TFR) cells] are thought to play central role[26]. Interestingly, recent reports documented that diabetic patients have cellular and molecular indicators of increased presence of circulating TFH cells[27,28]. We (unpublished data) and others[29] also found that these indicators are present in the peripheral blood prior to disease onset in AAb-positive (AAb^+) non-diabetic individuals, suggesting that TFH cells might contribute to AAb pathogenesis and T1D development.

Genetic susceptibility to T1D is defined by more than 10 genetic loci[30-32]. The most important genetic regions are: The *HLA* region, a critical susceptibility locus for many human autoimmune diseases[33,34], the *Insulin* gene, whose susceptibility resides in a variable number of tandem repeat polymorphisms in the promoter region of the gene[35-37], the *CTLA-4* gene, involved in negative regulation of immune responses[38,39], and importantly, the *PTPN22* gene, which encodes the lymphoid protein tyrosine phosphatase (LYP) an important negative regulator of TCR signaling, that is also involved in BCR and innate immune signaling[40-43]. The most convincing evidence that environmental factors play a major role in influencing T1D development derives from studies in monozygotic (MZ) twins where disease concordance is approximately 50%[44]. Viruses, vaccines, toxins and dietary factors (*e.g.*, breast feeding *vs* cow's milk) have been suspected for the increase of T1D incidence in developed countries[45-49]. However, the mechanism by which they activate the autoimmune process is unknown and they are thought to modify susceptibility by affecting the T cells' epigenome[50,51].

PTPN22

PTPN22 is the strongest non-HLA gene associated with the onset of T1D and other autoimmune diseases[7]. *PTPN22* encodes a non receptor protein tyrosine

phosphatase (PTP) which is expressed in hematopoietic cells. The PTP encoded protein, named LYP, consists of three domains: An N-terminal catalytic domain, an interdomain region and a C-terminal domain, characterized by the presence of a proline-rich region (P1-P4), that is important for the interaction with other proteins (reviewed in[7,8]).

PTPN22 plays a key role in regulating innate and adaptive immune responses. PTPN22 by enhancing pattern recognition receptors (PRRs) signaling, drives the activation of myeloid cells and promotes type 1 interferon (IFN) production. Specifically, PTPN22 associates with the TLR signaling molecule TRAF3 to promote its ubiquitination and thus the activation of IRF3 and IRF7 and the production of type 1 IFN[52]. PTPN22 dampens T-cell activation by restricting signalling downstream of the TCR. It dephosphorylates positive regulatory tyrosine residues in Src family kinases including ZAP-70 and Lck interacting with the C-terminal Src kinase (CSK) through its P1 motif[53-55] (reviewed also in[56]).

An allelic variant of *PTPN22* confers susceptibility to T1D[2-4] and other autoimmune diseases, like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[57-59]. This polymorphism is characterized by a single aminoacid substitution: An Arg (R) is replaced by a Trp (W) at position 620. This is a nonconservative variation of a residue within the P1 motif, which as we mentioned above, is critical for interacting with CSK. As a consequence, the predisposing autoimmunity variant R620W exhibits reduced interaction with CSK that leads to a further reduction of TCR signaling rendering T cells hyporeactive[60,61].

The PTPN22 autoimmune predisposing allelic variant influences B cells leading to reduced BCR signaling and increased resistence to apoptosis. Furthermore, the allelic variant R620W induces an up-regulation of various genes belonging to the BCR, CD40 and Toll-like receptor (TLR) signaling pathways that converge on nuclear factor-κB (NF-κB)[62,63]. As a consequence, increased survival of transitional and naïve B cells was observed[64]. PTPN22 R620W carriers contained increased frequencies of circulating transitional and anergic autoreactive B cells[63,65]. These alterations in the composition of the B cell pool were also characteristic of T1D patients, who also displayed higher frequencies of autoreactive clones[62].

The murine homologue of PTPN22 has a structure similar to the human protein and plays important roles in immune responses. Several mouse models were generated in order to understand the mechanism by which PTPN22 contributes to autoimmunity[56,66]. In mice, the allelic variant R619W is the equivalent of R620W in humans. The possibility to study PTPN22 R619W-expressing mouse models has allowed us to make direct comparisons between the human PTPN22 R620W allelic variant and the murine orthologue. For example, PTPN22 R619W knock-in mice reproduce many aspects of the human predisposing allelic variant including the increase of peripheral T effector/memory cells and autoreactive B cells[67-69], the reduction of circulating mature B cells[67,69] and the increase of transitional B cells[63,67] (also reviewed in[66]). These findings strongly suggest that the mouse orthologue could significantly reproduce the autoimmune risk effect of the human PTPN22 susceptibility allele. Below we discuss the role of PTPN22 in islet-specific autoimmunity as addressed by mouse studies.

PTPN22 IN ANIMAL MODELS OF T1D

The use of T1D animal models like the non-obese diabetic (NOD) mouse model, have helped us understand a lot about the pathogenesis of autoimmune diabetes[70,71]. These models serve to address how autoimmune predisposing genes like PTPN22 alter the immune system leading to T1D. Several mouse models including PTPN22 knock-out[72-75], PTPN22 R619W knock-in[67,68,76], PTPN22 R619W transgenic[77], PTPN22 knock-down[78] and PTPN22 WT transgenic[79] have been created on autoimmune-prone (like the NOD) or resistant genetic backgrounds to address the role of PTPN22 in autoimmunity. Whereas many of these studies showed a clear association between PTPN22 and autoimmunity, others supported the opposite. These controversial results indicated that the effect of PTPN22 on peripheral tolerance highly depends on the genetic background of the animal model employed, suggesting that other genes relevant for autoimmune predisposition play important role. These results also highlighted the complex role that PTPN22 plays in immune tolerance.

In the study where PTPN22 knock-out mice were described for the first time, lymphoproliferation, enlarged GCs and expansion of memory–phenotype cells were found[72]. However, no AAbs were produced nor spontaneous autoimmunity developed[72]. Aged PTPN22 knock-out mice exhibited increased numbers of TFH cells that support spontaneous GC formation and activity[75]. Interestingly, in a study where PTPN22 R619W knock-in mice were generated, anomalies comparable to those described in PTPN22-deficient mice were seen[68]. Also in this model no signs of spontaneous autoimmunity were observed[68]. This suggested that the autoimmune predisposing allele of PTPN22 represents a loss-of-function mutation. Interestingly, in a different report where a different PTPN22 R619W knock-in mouse strain was generated, spontaneous autoimmunity characterized by production of AAbs and cell infiltrates in multiple tissues (but not in pancreas) were seen[67]. In contrast to the first PTPN22 R619W knock-in mice however, which were generated on C57BL/6 (B6) autoimmune-resistant background, the PTPN22 R619W knock-in mice were placed on a mixed (B6x129) genetic background. Thus, these two studies suggest that the effect which PTPN22 has on the immune system is strongly dependent on the presence of other "modifier genes" present in the genetic background.

Table 1 Summary of mouse models where the role of protein tyrosin phosphatase non-receptor 22 in type 1 diabetes incidence has been directly or indirectly addressed

	Genetic background	Spontaneous autoimmunity[1]	AAbs	T1D	B cells	T cells	Ref.
PTPN22 knock-out	C57BL/6	No	No	No	intact	↑Memory/effector number ↑Treg number and function ↑TFH number and function ↑Tr1 number and function	[72-75,84]
PTPN22 knock-out	RIP-LCMV (B6)	No	No	Exacerbated	Not examined	↑Effector function	[81]
PTPN22 R619W knock-in	C57BL/6	No	No	No	Not examined	↑Memory/effector number	[68]
PTPN22 R619W knock-in	C57BL/6 x 129	Lupus-like disease	Yes	No	↑Transitional and Self-reactive	↑Memory/effector number	[67]
PTPN22 R619W transgenic	C57BL/6	No	No	No	Not examined	No differences	[77]
PTPN22 knock-down	NOD	No	No	Protected	Activation	↑Treg number and function	[78]
PTPN22 transgenic	NOD	No	No	Protected	Not examined	↓Memory/effector number	[79]
PTPN22 R619W knock-in	NOD	No	↑↑	Exacerbated	Not examined	Not examined	[76]

[1]Other than T1D. T1D: Type 1 diabetes; AAbs: Autoantibodies; Treg: T regulatory cell; PTPN22: Protein tyrosin phosphatase non-receptor 22; TFH: T follicular helper.

The role of PTPN22 in T1D was directly addressed by employing the NOD mouse model. Unexpectedly, NOD mice where PTPN22 expression was targeted by a knock-down genetic approach were protected from autoimmune diabetes[78]. Surprisingly, PTPN22 transgenic NOD mice that overexpressed PTPN22 were also protected from T1D[79]. Thus, either downregulation or overexpression of PTPN22 had a protective effect from T1D in NOD mice. PTPN22 knock-down in NOD mice resulted in T1D prevention possibly because of a dominant effect of PTPN22 on the T regulatory cell (Treg) compartment. As it was shown in several mouse models of diverse genetic background, the number and functionality of Treg cells increase when PTPN22 levels reduce[73,74,78]. On the other hand, transgenic NOD mice over-expressing PTPN22 were protected from T1D due to effects of PTPN22 on the effector T cell compartment, which showed reduced activation[79]. Instead, Treg development, differentiation and suppressive activity in PRPN22 overexpressing mice were similar to control[79]. These data suggest that whereas reduction in PTPN22 levels affects the Treg compartment, PTPN22 overexpression modifies the effector T cell compartment. In both cases the end result is protection from T1D. Additional experiments with conditional overexpression or downmodulation of PTPN22 and its variant, murine cell transfers and bone marrow chimeras could clarify these discrepancies. Nevertheless, these studies underline that if PTPN22 is selected as therapeutic target, caution should be taken in directing the drug to the correct cellular compartment.

More recently, PTPN22 R619W mutant NOD mice were generated in order to directly address the effect of the murine ortholog of R620W allele on T1D incidence. In contrast to PTPN22-knocked down mice, PTPN22 R619W NOD mice showed accelerated T1D and increased prevalence and elevated titer of insulin AAbs, suggesting an early loss of tolerance to insulin[76]. Thus, these findings suggest that the R619W variant possibly is not a loss-of-function variant.

To further understand the role of PTPN22 in T1D pathogenesis, our group employed a mouse model of virally-induced autoimmune diabetes (RIP-LCMV), which also served to address the role of PTPN22 on antiviral immunity[80]. RIP-LCMV PTPN22-deficient mice were more susceptible to diabetes compared to control mice[81]. Lack of PTPN22 altered the generation and function of effector-memory viral-specific T cells in an antigen-specific manner[81]. Our follow-up studies showed that PTPN22 plays central role in T-cell clonal expansion and effector function during acute infection; it promotes antigen-driven responses by positively regulating interferon signaling in T cells[82]. Thus, we identified a novel role of PTPN22 in T1D triggered by an acute viral infection and determined the role of PTPN22 in antiviral immunity.

We also explored the role of PTPN22 in pancreatic islet transplantation, which is one of the most promising approaches to cure T1D[83]. By employing a mouse model of acute allograft rejection, we found that PTPN22-deficient mice generate higher number of alloreactive T cells compared to control mice, but reject grafts with similar kinetics[84]. This was due to an increase of Treg and also T regulatory type 1 (Tr1) cells. In addition, a tolerogenic treatment known to induce transplant tolerance in C57BL/6 mice *via* Tr1 cell generation was more effective in PTPN22-deficient mice because it augmented the number and functions of both Tr1 and Treg cells[84]. Thus, lack of PTPN22 strengthened transplant tolerance to pancreatic islets, suggesting it could serve as therapeutic target to boost transplant tolerance.

Our group also investigated how PTPN22 affects the generation of Foxp3 Treg and T helper type 1 (Th1) cells. From *in vivo* and *in vitro* studies using PTPN22 knock-out mice we found that PTPN22 plays a key role in Treg induction and acts mainly through modulating the threshold of T cell activation. CD4 T cells from PTPN22 knock-out mice showed increased sensitivity to TCR activation and subsequently increased FOXP3

expression at low levels of stimulation[85]. However, FOXP3 expression was reduced at optimal-to-high levels of activation. Furthermore, we found that the absence of PTPN22 altered Th1 cell differentiation only at low levels of T-cell activation. These results underline the dual role PTPN22 has on determining Treg *vs* Th1 cell induction[85].

Taken together, several animal studies have examined the role PTPN22 on predisposing to autoimmunity and particularly T1D. Results so far corroborate with the notion that the immunomodulatory effects of PTPN22 are complex and suggest that PTPN22 may promote or inhibit autoimmunity depending on the genetic background and experimental setting.

CONCLUSION

The PTPN22 R620W allelic variant is associated with T1D and is considered the most important non-HLA predisposing gene. As detailed above and summarized in Table 1, using a number of murine models, investigators have started to decipher the role of PTPN22 in immune tolerance to pancreatic antigens. Because PTPN22 impacts multiple cells lineages it will be difficult to find the key cell subset or molecular mechanism by which PTPN22 breaks self tolerance unless advanced lineage-specific knock-in or deletion systems are employed. Importantly, the effect PTPN22 imparts on the immune system is strongly influenced by other genetic variants. In this review we focused on *PTPN22* and its role on islet-specific autoimmunity highlighting that targeting this protein may serve as possible future strategy to prevent T1D and perhaps other autoimmune diseases.

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MINIREVIEWS

Saponins as adipokines modulator: A possible therapeutic intervention for type 2 diabetes

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Abstract

Development of type 2 diabetes has been linked to β-cell failure coupled with insulin resistance and obesity. Adipose tissue, known as the fat store, secretes a number of hormones and proteins collectively termed adipokines some of which regulate insulin sensitivity. Dysregulation in the secretion of adipokines has been linked to insulin resistance and type 2 diabetes. In this review, we summarized evidence of the role of adipokines with focus on leptin, adiponectin, adipsin, visfatin and apelin in the pathogenesis of type 2 diabetes and discussed the potential of saponins to modify the ill-regulated adipokines secretions, which could promote the use of this class of phytochemicals as potential antidiabetics agents.

Key words: Adipokines; Adipose tissue; Insulin resistance; Antidiabetic; Obesity

Core tip: β-cell dysfunction and insulin resistance are linked to type 2 diabetes. Adipokines produced from adipose tissues regulate glucose homeostasis and insulin sensitivity. Dysregulation of adipokines are linked to insulin resistance and disruption of glucose Homeostasis. Saponins modulate the activity of some adipokines hence may serve as therapy for treatment of type 2 diabetes.

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INTRODUCTION

The Adipose tissue is the major origin of fatty acids in the postprandial fasting state for energy use and heat production[1]. Its accumulation, particularly the white adipose tissue (WAT), has been reported to be the factor responsible for obesity, which has been associated to type 2 diabetes and cardiovascular disease[2]. The statistic of individuals suffering from type 2 diabetes is growing worldwide and based on data from International Diabetes Federation, about 415 million people are affected by this metabolic but deadly disease, contributing to an explosion in type 2 diabetes linked health problems. Due to high rate of morbidity and mortality, type 2 diabetes is considered one of the major public health problem in many parts of the word[3].

Nowadays, adipose tissue is known to serve as endocrine organ that secrete pro- and anti-inflammatory mediators including adipokines, which are cell-signaling proteins that function as hormones[4]. Of particular importance is the ability of adipokines to function as classic circulating hormone that communicate with adipose tissue itself as well as other organs like muscle, liver, brain and the immune system[5]. It should be stressed that these adipokines are secreted to modulate inflammation and insulin resistance.

Insulin resistance is key to evolution of type 2 diabetes mellitus, which is regarded epidemic and culminating in high cardiovascular disease risk and death rate. Therefore, an in-depth knowledge of mechanisms implicit in insulin resistance is needful to fight the widespread occurrence of type 2 diabetes and their associated diseases[3]. Obesity's contribution to type 2 diabetes has been linked to dysregulation of adipokines (*i.e.*, improper production of adipokines by adipose tissue) and glucose uptake[6].

Increasing data have opened our understanding on adipose tissue over the past few decades giving us a clear picture about adipose tissue not only being an inert excess fat storage depot but also a dynamic endocrine organ secreting a wide range of bioactive protein secretions[7,8]. As mentioned earlier, adipokines or adipocytokines are peptides or cytokines that are secreted by adipose tissue. The adipokines list increases yearly, as both novel and existing adipokines secreted by adipose tissue are reported from time to time[8]. Adipokines play a substantial role in the maintenance of adipogenesis, chemo attraction of immune cells into adipose tissue, adipocyte function *via* autocrine/paracrine signaling, regulating appetite, energy expenditure and spontaneous activity, insulin sensitivity and energy metabolism in the brain and peripheral target tissues[9,10]. Some of the biologically active protein secretion of the adipocytes includes adiponectin, adipsin, leptin, resistin, apelin, retinol binding protein 4 (RBP4), vaspin, hepcidin and visfatin while the cytokine secretions are tumor necrotic factor-alpha, interleukin-6 and monocyte chemoattractant protein-1[11] (Figure 1).

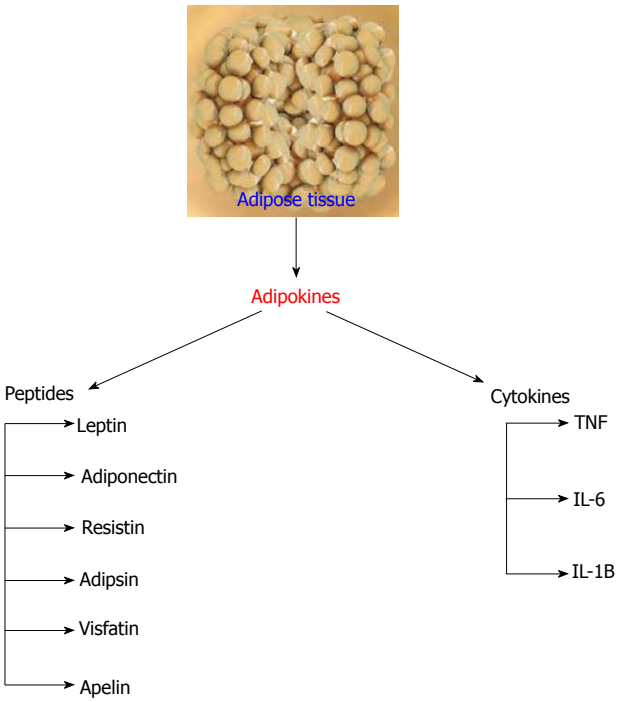

Figure 1 Peptides and cytokines secretion (adipokines) of the adipose tissue. TNF: Tumor necrotic factor; IL: Interleukin.

In the past few years, particular attention has been paid to finding natural products and/or plants derived chemicals with the potential to improve obesity (by suppressing appetite, retarding body fat accumulation and improving weight loss) and glucose uptake by modifying adipokines[12-15]. Saponins are steroid or triterpenoids glycosides found in many plants and plant products. They exhibit a variety of pharmacology activities including antidiabetic, hypocholesterolaemic, anticarcinogenic, and hypoglycaemia among others[16-18]. In this review, our focus shall be on the mechanisms linking adipokines to type 2 diabetes and discuss the ability of saponins to modulate adipokines thereby improving insulin sensitivity.

ADIPOKINES IN INSULIN RESISTANCE

A substantial risk factor for type 2 diabetes is obesity because it has been connected to insulin resistance. The diminished potential of tissues to react to insulin activity is referred to as insulin resistance. Adipose tissue is one of the tissues that respond to insulin action by storing triglycerides through some mechanisms which include enhancement of differentiation of pre-adipocytes to adipocytes, enhancing the intake of glucose and fatty acids derived from circulating lipoproteins and

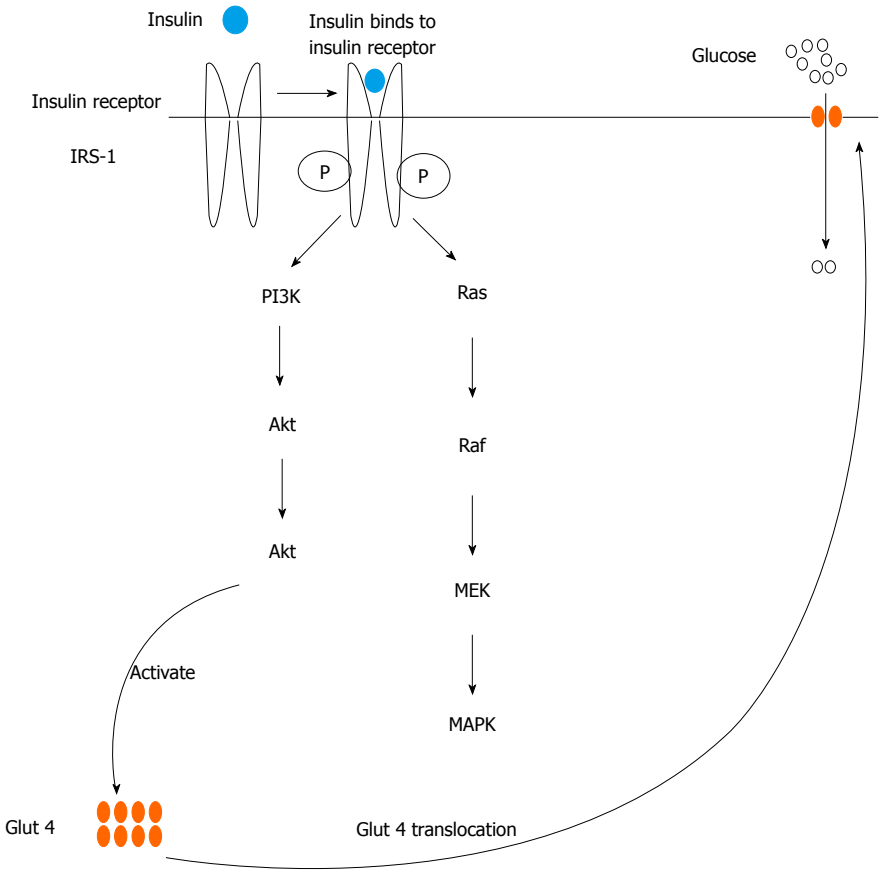

Figure 2 Insulin signaling leading to increase in adipocyte glucose uptake. PI3K: Phosphatidylinositol 3-kinase; Akt: Protein kinase B; MAPK: Mitogen activated protein kinase; Glut 4: Glucose transporter 4; Raf: Raf family of serine/threonine kinases; Ras: Superfamily of small GTPases; MEK: MAPK kinase; IRS-1: Insulin receptor substrate 1.

lipogenesis in mature adipocytes, and inhibiting lipid breakdown (lipolysis)[19]. Initiation of insulin signaling starts by binding of insulin to its receptor located on the cell membrane. The binding leads to activation of insulin receptor substrate (IRS) proteins by phosphorylation thereby activating two main associated signaling pathways: Namely the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway. The most important pathway for most metabolic actions of insulin is PI3K-AKT/PKB. The phosphorylated IRS-1, by the insulin receptor, triggers PI3K by binding to its SH2 domain. PI3K produces phosphatidylinositol-(3,4,5)-triphosphate (PIP3), which is a lipid second messenger that triggers many phosphatidylinositol-(3,4,5)-triphosphate-dependent serine/threonine kinases, including AKT/PKB. These downstream signaling pathways of insulin result in the mobilization of glucose transporter 4 (Glut 4) to the plasma membrane from the cytosol, resulting in increased adipocyte glucose uptake (Figure 2). The MAPK pathway that is the second pathway associated with IRS-1 phosphorylation is not associated metabolic actions of insulin. It is rather involved in inducing mitogenic and growth effects of insulin. Insulin also has anti-lipolytic effect in adipose tissue, through PI3K activation which stimulates phosphodiesterase-3 causing hydrolysis of more adenosine 3′,5′-cyclic monophosphate in adipocytes, thereby limit the mobilization of fatty acids from adipose tissue[19].

One of the mechanisms to explain the high risk of type 2 diabetes with obesity is as a result of defect in blood level of adipokines on metabolic tissues[20]. Study has suggested a probable role of adipocytes in the progression of insulin resistance. Adipose tissue releases free fatty acids (FFAs) and various adipokines that have been implicated in unnatural insulin signaling. Study has demonstrated that the enlargement of adipose tissue depots leads to obesity causing dysregulation in adipokine secretion, typifying the potential pathophysiological link between adipose tissue secretions (adipokines), obesity and type 2 diabetes[21]. Compositional changes in obese state lead to dysregulation in secretion of adipocyte-secreted hormones (adipokines). Adipose tissue secrets many adipokines like RBP4, leptin, resistin, vaspin, visfatin, hepcidin, adiponectin and inflammatory cytokines which regulate insulin sensitivity, immune response, cardiovascular function, and many physiological processes[22]. A strong correlation exists with level of circulating adipokines and signaling pathways modulated by insulin (such as JAK2/STAT3, MAPK, PI3K and AMPK pathways), suggesting a link between adipokines and insulin action.

Adipokines such as adiponectin and leptin, visfatin, apelin are now known to modify insulin sensitivity and/or secretion which are the two major events that occur in the evolvement of type 2 diabetes. For the purpose of this review, we will focus our attention on leptin, adiponectin, adipsin visfatin and apelin. Particularly, we will discuss their mechanism of action in regard

to insulin resistance and the potential of saponins to modulate peptides adipokines.

Leptin and insulin sensitivity

Leptin is an endogenous sensing factor that provides a critical link between the environment, metabolism, and immune function[22]. It plays vital role in the metabolic regulation of satiety, appetite, food intake, activity and energy expenditure. The relationship of leptin with insulin resistance, obesity and cardiovascular disease has been extensively studied since its discovery in 1994. As mentioned earlier, obesity, which is considered a major public health problem, is often linked with type 2 diabetes mellitus, cardiovascular diseases as well as cancer. These diseases have been linked to a lowered reactivity for leptin, an adipocyte hormone that is principally secreted by the WAT to targets specific receptors in the arcuate nucleus of the hypothalamus in order to regulate food intake and energy expenditure. Leptin was originally thought to act only as a satiety factor but the presence of OB-R leptin receptors in almost all tissues suggest the pleiotropism of leptin in all tissues expressing leptin receptors. The actions of leptin are mediated *via* actions on leptin receptors (LepRs) generally expressed by neurons in the central nervous system (CNS)[23]. Leptin receptors (OB-R) activation stimulates several intracellular signaling pathways implicated in insulin sensitivity such as the PI3K, JAK2/STAT3, MAPK, and AMPK pathways, IRSs[24] for review see[25,26].

Saponin's effect on leptin: Saponins have been implicated in regulation of energy metabolism through activation of AMPK[27,28]. In addition, most of the signaling pathways (JAK2/STAT3, MAPK, PI3K and AMPK) being modulated by leptin are also modulated by saponins[29-31]. Several studies on the effect of saponins on leptin have been documented[32]. While some recorded increase in serum leptin concentration with saponin administration[31,33], others documented decrease in serum leptin concentration following saponin administration[34,35].

In a study, Yang *et al*[35] reported that *Panax notoginseng* saponins demonstrated anti-hyperglycemic and anti-obese activities as a result of improved insulin and leptin sensitivity. Tea saponin treatment was shown to reduce the protein levels of pro-inflammatory cytokines [tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and/or IL-1β] and nuclear factor-κB signaling (phosphorylated inhibitory-κB kinase and phosphorylated inhibitory-κBα) in adipose tissue and the liver[36]. The anti-inflammatory effect of tea saponin was associated with improved glycemic status in the treated animals, which was evidenced by improved glucose tolerance, homeostasis model assessment, and fasting plasma insulin. In the hypothalamus, tea saponin decreased both pro-inflammatory cytokines and inflammatory signaling in the mediobasal hypothalamus. Tea saponin treatment also enhanced the anorexigenic effect of central leptin administration, restored leptin phosphorylated signal transducer and activator of transcription-3 (p-STAT3) signaling in the arcuate nucleus, making tea saponin an anti-obesity and anti-diabetic agent. Other plants whose saponins effects have been probed on leptin are *Yucca schidigera*[32]. Based on the aforementioned information, saponins appear to be an activator of AMP-activated protein kinase (AMPK), which is a key regulator of energy balance and fat metabolism and PI3K signaling, leading to improve insulin sensitivity. Hence, saponin may be a potential anti-obesity agent by reducing insulin resistance and improving insulin sensitivity.

Adiponectin and insulin sensitivity

Adiponectin is a protein hormone (adipocyte hormone) modulating a number of metabolic processes such as fatty acid oxidation and glucose regulation[27,37]. It plays a crucial role in the evolution of insulin resistance and atherosclerosis. The concentration of circulating adiponectin is high in normal subject but lower in obese subjects than in lean subjects. Adiponectin is negatively correlated with adiposity. Its level is also reduced in insulin resistance and type 2 diabetes. A reduction in adiponectin level occurs prior to the onset of type 2 diabetes and oral administration of adiponectin is generally followed by decrease blood glucose levels which culminates in increased insulin sensitivity (for reviews, see[38,39]. Data from animal studies have linked decrease expression of adiponectin to some degree of insulin resistance thereby linking hypoadiponectinaemia to insulin resistance. Increase fatty acid oxidation and hepatic glucose production inhibition have been put forward as mechanism of enhancement of insulin sensitivity by adiponectin[40]. AdipoR1 and AdipoR2 are characterized adiponectin receptors and they contain 7 transmembrane domains, with different structure and function. Both AdipoR1 and AdipoR2 are predominant in in the skeletal muscle while AdipoR2 is primarily expressed by liver[41]. AdipoR1 and AdipoR2 mediate the antidiabetic metabolic effect of adiponectin, and their expression are repressed in obesity-linked insulin resistance[38,39].

Saponin's effect on adiponectin: Accumulating evidences from the literature indicate that saponin treatment increases adiponectin level, and this effect might play an important role in enhancement of insulin sensitivity by saponins[17,27,42]. Duan *et al*[43] reported that chikusetsu saponin increased adiponectin level and enhanced neuronal AdipoR1 as well as downstream molecules of adiponectin including AMPK, and glycogen synthase kinase 3 beta (GSK-3β) expression, in a concentration-dependent manner in diabetic mice. Platyconic acid is a saponin from *Platycodi radix* that potentiated the expression of adiponectin in adipose tissue leading to improved insulin signaling[42]. Likewise,

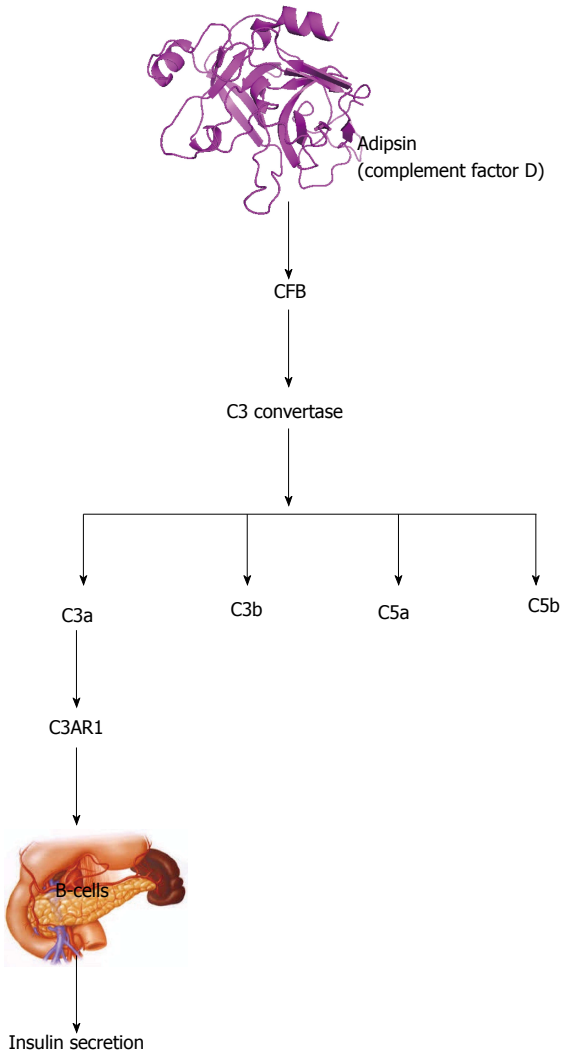

Figure 3 Adipsin and insulin secretion in beta cell. Adipsin potentiates insulin secretion through cleavage of CFB to form C3 that is hydrolyzed to form C3a. C3a activates C3AR1, which acts on B-cells of the pancreas to secret insulin. CFB: Complement factor B.

saponins from *Helicteres isora* increased the expression of adiponectin[17]. Other saponins which exhibited increased expression of adiponectin include saponins isolated from *Astragalus membranaceus*[44] and Ilex *paraguariensis*[45].

Adipsin and insulin sensitivity

Adipsin was the first adipokine described[46] and is one of the major proteins of adipose cells that inversely correlate with many animal models of obesity and diabetes[46]. Later this adipokine identified to be complement factor D[47-49], which catalyzes the rate-limiting step of the alternative pathway of complement activation[50]. Since then, adipsin has been shown to play pivotal roles in models of ischemia reperfusion and sepsis[51-53].

Adipsin stimulates glucose transport enhancing triglyceride accumulation in fats cells and also inhibits lipolysis[54]. The adipsin-acylation stimulating protein (ASP) system is involved in the regulation of triglyceride metabolism in adipocytes. This system increases triglyceride synthesis rate in adipocytes by translocation of glucose transporters from intracellular vesicles to the plasma membrane, enhancing specific membrane glucose transport[27,55].

Recently, the relationship between the immune system and adipose tissue has linked complement biology to pathogenesis of type 2 diabetes. This can be explained at least in part to the fact that certain proteins of the complement pathway such as adipsin are preferentially expressed in the adipose tissue and are dis-regulated in models of obesity and diabetes[53]. Adipsin was recently identified as one of the most abundant and specifically expressed adipose proteins that links fat cells and obesity to Beta cell function[53]. It can increase insulin secretion by producing the peptide complement 3a (C3a).

Adipsin splits complement factor B in the alternative complement pathway, hence catalyzing the formation of C3 convertase, contributing to a hydrolysis cascade that produces various complement fragments including complement 3a (C3a), C3b, C5a and C5b[48]. C3a potentiates insulin secretion by interacting with C3AR1 to act on Beta cells (Figure 3) only during hyperglycemia and does not induce Beta cells to release insulin at low glucose level[56].

Saponin's effect on adipsin: Bhavsar *et al*[17] reported that saponins from *Helicteres isora* significantly increase the expression of adipsin when compared with control db/db mice. Zhang *et al*[57] also established the link between *Panax notoginseng* saponins and complement factor 3 (C3). The ability of saponin to stimulate adipsin and C3 brings to light the beneficial role of saponins in improving insulin sensitivity and hyperglycemia.

Visfatin and insulin sensitivity

Visfatin also known as nicotinamide phosphoribosyl-transferase (NAMPT), or pre-B-cell colony-enhancing factor 1 (PBEF-1) is an adipokine mainly synthesized and secreted in visceral fat (WAT) hence its name "visfatin"[58]. It is produced as a result of adipocyte differentiation and its potential to lower blood glucose is as a result of its nicotinamide phosphoribosyl transferase activity[59]. Visfatin possess insulin mimetic effects through enhancement of glucose uptake by myocytes and adipocytes and suppression of hepatocyte glucose production/release[11,60]. Visfatin also exert its effect on insulin transduction pathway through induction of tyrosine phosphorylation of insulin receptors 1 and 2, activation of phosphatidylinositol-3 kinase (PI3K), protein kinase B (AKT) and MAPK. Visfatin has the same affinity as insulin for insulin receptor but its binding to insulin receptor occur at a different site. Brown *et al*[61] demonstrated that visfatin is able to regulate insulin secretion and insulin receptor signaling in beta-cells of the pancreas. More recently, Gouranton *et al*[62]

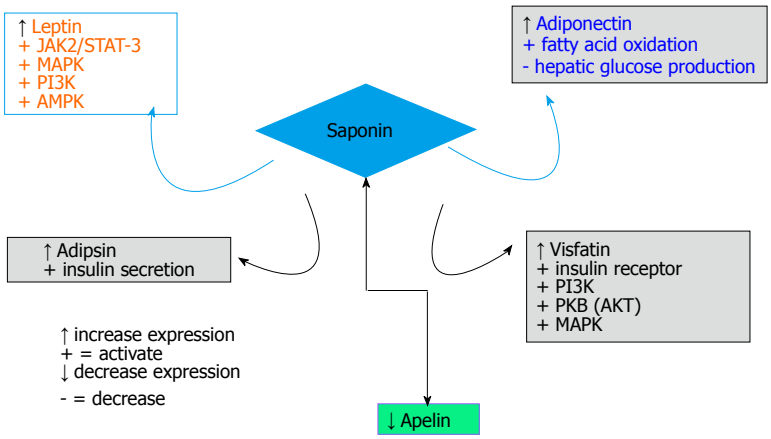

Figure 4 Modulation of adipokines (peptides) by saponin. Saponin increases the expression of leptin, adiponectin, adipsin, visfatin but reduces the expression of apelin. PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase B; MAPK: Mitogen activated protein kinase; AMPK: 5'AMP-activated protein kinase; STAT3: Signal tranducer and activator of transcription 3; JAK2: Janus kinase 2.

demonstrated that visfatin is involved in TNFα-mediated insulin resistance through NDA^{+}/Sirt1/PTP1B pathway in 3T3-L3 adipocytes.

Saponin's effect on visfatin: Increasing evidence has shown that saponins act the same way as visfatin by activating PI3K, protein kinase B (AKT) and MAPK suggesting that saponins can regulate insulin transduction pathway[17,27,63]. Macrostemonoside A, a steroidal saponins from *Allium* genus increased the synthesis as well as release of visfatin in 3T3-L1 adipocytes and elevated mRNA levels of this adipokine in a dose- and time-dependent mode[64,65].

Apelin and insulin sensitivity

Apelin, a 36 amino-acid peptide has been characterized in a variety of tissues, such as CNS with high expression in the hypothalamus, stomach, heart, skeletal muscle, and WAT. It is an endogenous ligand of the G-protein-coupled receptor (APJ)[65,66]. The G protein-coupled receptor APJ and its connected ligand, apelin, are widely expressed all through human body. They are linked to different key physiological processes including cardiovascular functions, fluid homeostasis, angiogenesis and energy metabolism regulation. The serum level of apelin is directly proportional to insulin resistance[67-69] and liver cirrhosis. Inflammation and oxidative stress have been shown increase plasma level of apelin.

One of the first observed effect of apelin linked to glucose metabolism, aside that of insulin secretion is its ability to lower glucose level in fasted states and during *in-vivo* mice model of glucose tolerance test. This effect is mainly due to enhanced glucose uptake in target tissues such as adipose tissue and skeletal muscle[70,71].

Data from *in vivo* study revealed that reduced expression of apelin in adipocyte and lower serum concentration might contribute to enhanced insulin sensitivity that is significantly independent of weight loss through an unknown mechanism.

In vitro experiment using C2C12 muscle cells showed that apelin enhanced glucose transport through AMPK pathway. Also apelin increased muscle Akt phosphorylation in both *ex vivo* and *in vitro* studies[70,72]. Interestingly, apelin triggers glucose uptake in muscle of obese as well as insulin-resistant mice ultimately leading to enhanced insulin sensitivity[70,71].

Saponin's effect on apelin: Only one study reported the potential beneficial effect of saponin on apelin. Xiu-Juan *et al*[73] demonstrated that-saponins from *Astragalus membranaceus* decrease the expression of Apelin/APJ mRNA in the high glucose group when compared to control.

MODULATION OF ADIPOKINES (PEPTIDES) BY SAPONIN

We have demonstrated in this review that saponin modulates leptin, adiponectin, adipsin, visfatin and apelin (Figure 4). Leptin and visfatin activation by saponin may be the link between saponin and insulin signaling. Earlier studies have documented the potential of saponin to activate PI3K and AKT[17], the activation of PI3K and AKT by saponin may be the downstream signaling resulting from leptin and visfatin activation.

Primarily, hyperlipidemia, serum triglycerides and FFA are elevated in type 1 and type 2 diabetes but plasma FFA are elevated in obese subjects. An elevated plasma level of FFA has been linked to increase insulin resistance in muscle and liver. One of the therapeutic approaches for type 2 diabetes has been to lower circulating level of FFA[74]. Activation of adiponectin by saponin could increase fatty acid oxidation and inhibit hepatic glucose production thereby lowering plasma FFA levels. Increase expression of adiponectin by saponin could be one of the mechanisms of improving insulin sensitivity by saponin. Increase expression of adipsin by saponin (Figure 4) is also another way by which saponin can improve insulin sensitivity in type 2 diabetes.

CONCLUSION

This mini review has outlined the link between adipokines, insulin resistance and type 2 diabetes and

the ability of saponin to modulate peptide adipokines (leptin, adiponectin, adipsin, visfatin and apelin) leading to improved insulin sensitivity. Further insight into this area of developing saponin into a class of antidiabetic drug will be invaluable and of tremendous impact on the treatment and the early intervention and prevention of diabetes.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Vitamin D levels in subjects with or without chronic kidney disease among Veterans with diabetes in North East United States

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Abstract

AIM

To evaluate the prevalence of vitamin D deficiency and its relation to diabetes and kidney disease in Veterans residing in the North East United States (VISN 2).

METHODS

In this retrospective study, we used data from the computerized patient record system at Stratton Veterans Administration Medical Center at Albany, NY (VHA) for those patients who had 25-hydroxyvitamin D levels and 1,25 (OH) vitamin D levels measured between 2007 and 2010. We collected demographic information including age, sex, body mass index and race; clinical data including diabetes, hypertension and CAD; and laboratory data including calcium, creatinine and parathyroid hormone (PTH) (intact). Vitamin D deficiency is defined as a serum 25-hydroxyvitamin D level of less than 20 ng/mL (50 nmol/L), and insufficiency is defined as a serum 25-hydroxyvitamin D level of 20 to 30 ng/mL (50 to 75 nmol/L).

RESULTS

Data was available for approximately 68000 subjects. We identified 64144 subjects for analysis after exclusion of duplicates. Among them, 27098 had diabetes. The

mean age of subjects with diabetes was 68 ± 11 with a mean body mass index (BMI) of 32 ± 7 and duration of diabetes of 5.6 ± 3.2 years. The mean 25 (OH) vitamin D level among subjects with diabetes was 27 ± 11.6. There was no significant difference in 25 (OH) vitamin D levels between subjects with diabetes and glomerular filtration rate (e-GFR) < 60 compared to those with e-GFR ⩾ 60. As expected, subjects with e-GFR < 60 had significantly lower 1,25 (OH) vitamin D levels and significantly elevated PTH-intact. Of the 64144 subjects, 580 had end-stage renal disease. Of those, 407 had diabetes and 173 did not. Vitamin D levels in both groups were in the insufficiency range and there was no significant difference irrespective of presence or absence of diabetes. Subjects with vitamin D levels less than 20 ng/mL had a higher BMI and elevated PTH, and higher HbA1C levels compared to those with vitamin D levels more than 20 ng/mL.

CONCLUSION

We conclude that we need to keep a close eye on vitamin D levels in subjects with mild chronic kidney disease as well as those with moderate control of diabetes.

Key words: Vitamin D; Type 2 diabetes; Men; Chronic kidney disease; End stage renal disease

Core tip: This retrospective study evaluated the prevalence of vitamin D deficiency among Veterans in the North East United States, for those patients who had vitamin D levels measured between 2007 and 2010. The data collected include the data of 27098 subjects with diabetes with mean age of 68 and mean duration of diabetes of 5.6 years. There was no significant difference in 25 (OH) vitamin D levels between subjects with > glomerular filtration rate (e-GFR) < 60 and eGFR ⩾ 60 but with decreased levels of 1,25 (OH) vitamin D and elevated parathyroid hormone. Vitamin D levels did not differ between subjects with or without diabetes.

Yaturu S, Youngberg B, Zdunek S. Vitamin D levels in subjects with or without chronic kidney disease among Veterans with diabetes in North East United States. *World J Diabetes* 2017; 8(7): 346-350 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i7/346.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i7.346

INTRODUCTION

The prevalence and incidence of type 2 diabetes mellitus (T2DM) is increasing. As per the National Diabetes Statistics Report, 2014, 29.1 million people or 9.3% of the population have diabetes. Vitamin D plays an important role in calcium homeostasis and maintenance of optimal skeletal health. The vitamin D status is marked by the serum levels of 25-hydroxyvitamin D concentration[1]. Factors that influence circulating vitamin D levels include race, season, body mass index, and age[1]. Sources of vitamin D in humans include exposure to sunlight, diet, and dietary supplements. In the presence of vitamin D, calcium is actively absorbed from the small intestine. Vitamin D refers to vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol). Vitamin D deficiency can lead to rickets in children, osteomalacia in adults, myopathy and a variety of extra skeletal problems, including cardiovascular disease, infection, malignancy, and death[1-3]. Vitamin D from the dietary resources and sun exposure gets metabolized in the liver to 25-hydroxyvitamin D[1]. Vitamin D insufficiency affects almost 50% of the population worldwide[1,4]. McMurtry *et al*[5] reported that most Veterans living in nursing homes have vitamin D insufficiency. Later, the prevalence of vitamin D deficiency/insufficiency in long-term care patients at a Veterans Health Administration (VHA) hospital was reported to be as high. McMurtry *et al*[6] stated that 49%of them had sufficient vitamin D, 14% had insufficiency, and 37% had deficiency. The aim of this study was to evaluate the prevalence of vitamin D deficiency among Veterans with diabetes in North East United States. In addition we planned to compare vitamin D levels in subjects with diabetes with or without chronic kidney disease (CKD) and end stage renal disease (ESRD).

MATERIALS AND METHODS

Design

Single center, retrospective database study.

Study site

Stratton Veterans Administration Medical Center, Albany, NY, United States.

Information collected

Using the data base of the Veterans Health Administration (VHA) computerized patient record system (CPRS) at Stratton Veterans Administration Medical Center, we collected the data for those patients who had 25-hydroxyvitamin D levels and 1,25 dihydroxy vitamin D levels measured between 2007 and 2010, after the approval of the protocol both by the Institutional Review Board (IRB) and Research and Development (R and D) Committees at the VA Medical Center. We collected demographic information including age, sex, body mass index and race; clinical data including diabetes, hypertension and CAD; and laboratory data including calcium, creatinine and parathyroid hormone (PTH) (intact). The vitamin D levels were measured by immunoassay.

Definitions

Vitamin D deficiency is defined as a serum 25-hydroxyvitamin D level of less than 20 ng/mL (50 nmol/L), and insufficiency is defined as a serum 25-hydroxyvitamin D level of 20-30 ng/mL (50 to 75 nmol/L).

Statistical analysis

The statistical analysis performed include: Data is

Table 1 Clinical and biochemical parameters in all subjects with diabetes (n = 27098)

Parameter	Mean ± SD
Age	68 ± 11
BMI	32 ± 7
DM duration (yr)	5.6 ± 3.2
HTN (%)	94
Vit D	27 ± 11.6
Cre	1.38 ± 0.9
eGFR	66 ± 24
Calcium	9.5 ± 0.5
Glucose	142 ± 64
HbA1C	7.2 ± 1.4
PTH	126 ±121
1,25 Vit D	28 ± 16
IHD (%)	24

BMI: Body mass index; Dur of DM: Duration of diabetes; HTN: Hypertension; Vit D: Vitamin D; Cre: Creatinine; PTH: Parathyroid hormone; IHD: Ischemic heart disease.

Table 2 Clinical and laboratory parameters in subjects with diabetes with glomerular filtration rate < 60 compared to those with glomerular filtration rate ⩾ 60

	Mean ± SD eGFR ⩾ 60	Mean ± SD eGFR < 60	*P* value
Age	64.9 ± 11	72.5 ± 10	0
BMI	32 ± 7	31.5 ± 6.5	1.09E-16
Dur of DM	5.2 ± 3.2	6.33 ± 3	1.90E-165
HTN	92%	97%	2.10E-99
Vit D	27 ± 11.5	26.6 ± 12	0.751554
Cre	1.0 ± 0.16	2.02 ± 1.3	0
eGFR	81 ± 16	41.6 ± 13	0
Calcium	9.6 ± 0.46	9.4 ± 0.6	4.23E-97
Glu	141 ± 60	144 ± 68	4.25E-05
HbA1C	7.2 ± 1.46	7.25 ± 1.4	0.018789
PTH	64 ± 43	152 ± 133	1.20E-236
1,25 Vit D	34.8 ± 16	25 ± 14	1.30E-20

GFR: Glomerular filtration rate; BMI: Body mass index; Cre: Creatinine; HTN: Hypertension; Vit D: Vitamin D; PTH: Parathyroid hormone.

expressed as mean ± SD. Comparison of clinical and lab parameters of subjects with GFR < 60 and ⩾ 60 with diabetes is carried out. Comparison of clinical and lab parameters of subjects with vitamin D levels < 20 and > 20 with diabetes is carried out. Comparison of clinical and lab parameters of subjects with ESRD with or without diabetes was carried out.

RESULTS

Data was available for approximately 68000 subjects. We identified 64144 subjects for analysis after exclusion of duplicates. Among them, 27098 had diabetes. The mean age of subjects with diabetes was 68 ± 11 with a mean body mass index (BMI) of 32 ± 7 and duration of diabetes of 5.6 ± 3.2 years. The mean 25 (OH) vitamin D level among subjects with diabetes was 27 ± 11.6. The clinical and biochemical parameters of all the subjects with diabetes are shown in Table 1. The prevalence of vitamin D deficiency is 31% and insufficiency is 35%. We noted negative correlation of 25 (OH) vitamin D levels with BMI (r = - 0.12; P < 0.001), HbA1C (r = -0.14; P < 0.001), glucose (r = -0.12; P < 0.001), and PTH levels (r = -0.11; P < 0.0001). We noted that the 25 (OH) vitamin D levels did not correlate with age or duration of diabetes or creatinine. In subjects with decreased GFR, the vitamin D levels correlated with age. Comparison of the clinical and biochemical parameters of the subjects with eGFR < 60 and eGFR ⩾ 60 are shown in Table 2.

Subjects with hypovitaminosis D in comparison with patients with vitamin D sufficiency, had higher BMI (33 ± 7.0 *vs* 32 ± 6.0; P = 2.53E-08); higher HbA1C (7.4 ± 1.6 *vs* 7.0 ± 1.3; P = 2.5E-139); higher PTH values (142 ± 1074 *vs* 117 ± 119.0; P = 2.86E-89) as in Table 3. There were 580 subjects with ESRD. Among these subjects with ESRD, 407 had diabetes and 173 without diabetes. The comparative clinical and biochemical data of the subjects with ESRD are shown in Table 4 showing the differences between subjects with and without diabetes. In subjects with ESRD the 25 (OH) vitamin D levels correlated significantly with age.

DISCUSSION

There are several relevant features in the current study. The first notable point in our study is the high prevalence of vitamin D deficiency (31%) and insufficiency (35%) among subjects with diabetes. We noted that patients with hypovitaminosis D, compared to the patients with vitamin D sufficiency, had higher BMI, higher HbA1C and higher glucose levels. Our data is similar to the other reports[7] that correlation of low vitamin D levels with high body fat and glucose levels[7]. In our data base study, we noted a negative association of vitamin D levels with HbA1C indicating the association with glycemic control. This is similar to other studies. Studies on supplementation of vitamin D and calcium has been shown to improve insulin sensitivity in prediabetes[8] and beneficial effect on glycemic parameters in male type 2 diabetic patients[9], whereas no effect was noted on long-term glycemic control for T2DM in a South Korean study[10]. Studies in adults with prediabetes, who are at risk for type 2 diabetes, short-term supplementation of vitamin D though improved β cell function but had a marginal effect on attenuating the rise in HbA1C[11].

Comparison of the clinical and biochemical parameters of the subjects with eGFR < 60 and eGFR ⩾ 60 are shown in Table 2. Prevalence of vitamin D deficiency is common in chronic kidney disease, but lower intake was considered unlikely to be the cause[12]. In CKD, altered vitamin D metabolism leads to secondary hyperparathyroidism and CKD-mineral bone disease (CKD-MBD). Studies suggest that in patients with CKD, vitamin D deficiency was reported to be associated with increased cardiovascular related morbidity, mortality and all-cause mortality[13,14]; in both type 1 and 2 DM[15,16]. We noted that the 25 (OH) vitamin D levels did not correlate with age or duration of diabetes or creatinine.

Table 3 Clinical and laboratory parameters in subjects with diabetes mellitus with vitamin D levels < 20 and > 20

	Vit D < 20 Mean ± SD	Vit D > 20 Mean ± SD	*P*-value
Age	66 ± 12	68 ± 11	2.53E-08
BMI	33 ± 7	32 ± 6	5.09E-12
HTN (%)	93	90	NS
25, OH Vit D	14.2 ± 4	32 ± 9	0
Creatinine	1.4 ± 1.0	1.3 ± 0.8	4.60E-130
eGFR	67 ± 26	67 ± 24	7.96E-23
Calcium	9.5 ± 1.4	9.6 ± 1.8	1.20E-208
Glucose	148 ± 69	136 ± 56	1.20E-111
HbA1C	7.4 ± 1.6	7.0 ± 1.3	2.50E-139
PTH	142 ± 174	117 ± 119	2.86E-89

GFR: Glomerular filtration rate; BMI: Body mass index; HTN: Hypertension; Vit D: Vitamin D; PTH: Parathyroid hormone.

Table 4 Clinical and laboratory parameters in subjects with end stage renal disease and with or without diabetes mellitus

	Diabetes (407) Mean ± SD	Without diabetes (173) Mean ± SD	*P*-value
Age	67 ± 2	68 ± 4	0.0824
BMI	30.5 ± 2	25.2 ± 4	3.00E-13
HTN (%)	98	91	
25 (OH Vit D)	24 ± 1.4	24 ± 1.6	0.129
Creatinine	6.6 ± 1.1	7.1 ± 1.1	0.2588
eGFR	10 ± 2	9.1 ± 2.2	0.5871
Calcium	8.9 ± 0.1	8.9 ± 1.1	0.2059
Glucose	147 ± 12	104 ± 8	3.00E-47
HbA1C	6.8 ± 1.4	5.5 ± 0.4	9.29E-16
PTH	353 ± 305	346 ± 290	0.4339

GFR: Glomerular filtration rate; BMI: Body mass index; HTN: Hypertension; Vit D: Vitamin D; PTH: Parathyroid hormone.

Among 580 subjects with end stage renal disease (ESRD), 407 had diabetes and 173 did not. The prevalence of patients on renal replacement therapy for ESRD is increasing globally, diabetes being the leading cause; with an increase in prevalence of ESRD attributed to diabetic kidney disease increased by 2.5 fold in the last decade[17,18]. The relatively increased number of subjects receiving renal replacement therapy among subjects with diabetes is similar to the renal databases from United States and United Kingdom[17,18]. Vitamin D deficiency was common among subjects with ESRD on dialysis with no significant difference between subjects with or without diabetes. This is in contrast to the other published data[19]. Schiller and associates reported that vitamin D deficiency is significantly higher prevalence in patients on hemodialysis secondary to diabetic kidney disease and have a higher overall mortality than non-DM patients. They also suggested mandatory screening for vitamin D deficiency as an optimal risk reduction strategy[19].

The Third National Health and Nutrition Examination Survey (NHANES Ⅲ), vitamin D levels in the lowest quartile (< 17.8 ng/mL) was reported to be independently associated with increased all-cause mortality[20]. Regarding the micro and macrovascular complications and association with vitamin D deficiency, the literature has varied data. Subjects with diabetic neuropathy said to have lower vitamin D levels (81.5%) compared to those without neuropathy (60.4%)[21]. Regarding vitamin D levels in subjects with diabetic retinopathy, some authors report that the prevalence of diabetic retinopathy doubles at vitamin D level of less than 15.57 ng/mL[22], while others did not find any association between diabetic retinopathy and its severity and vitamin D insufficiency[23]. In the NHANES data, the prevalence of peripheral arterial disease is high among subjects with low vitamin D[24]. Some studies suggest improvement in HbA1C with vitamin D supplementation in subjects with T2D[25], while others did not notice significant improvement in glycemic control[26].

Limitations of this study: Information on supplementation of vitamin D or associated other medical problems that possibly might have led to vitamin D deficiency are not included into the data retrieved.

In conclusion, vitamin D deficiency is high among Veterans from the North East. Since vitamin D deficiency among subjects with diabetes is associated with higher BMI and higher HbA1C, it is important to screen the obese diabetics as well as moderate to poor glycemic control subjects as it can be easily supplemented. That may result in improvement in skeletal health in subjects with diabetes who have a higher risk for fractures.

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COMMENTS

Background

Vitamin D deficiency is reported to be common among subjects with diabetes. Similarly vitamin D is a common association in subjects with chronic kidney disease. Several reports in literature indicate vitamin D is common in North East United States.

Research frontiers

Since the data suggests that vitamin D deficiency is more common in subjects with poor control as well as those with higher body mass index (BMI), it is worthwhile look into the causes of poor glycemic control and supplementing vitamin D along with improving glycemic control.

Innovations and breakthroughs

The current data is nothing innovative or breakthrough. The data suggests that vitamin D deficiency is more common in subjects with poor control as well as those with higher BMI.

Applications

It is advisable to monitor vitamin D status in diabetes subjects with poor

glycemic control and or obesity.

Terminology

Vitamin D deficiency: Serum 25-hydroxyvitamin D level of less than 20 ng/mL (50 nmol/L); Vitamin D insufficiency: Serum 25-hydroxyvitamin D level of 20-30 ng/mL (50 to 75 nmol/L). CKD: Chronic kidney disease; ESRD: End stage renal disease.

Peer-review

The aim of this study was to evaluate the prevalence of vitamin D deficiency and its relation to diabetes and kidney disease in Veterans residing in the North East United States.

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ORIGINAL ARTICLE

Observational Study

Type 2 diabetes in a Senegalese rural area

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Author contributions: Duboz P and Macia E designed the study; Duboz P wrote the analysis plan, conducted the literature review and analysis, and wrote the first draft manuscript; Boëtsch G, Gueye L and Macia E reviewed the draft manuscript, provided critical comments and suggested additional analyses; Duboz P finalized the manuscript, which was subsequently approved by all authors.

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Abstract

AIM

To estimate the prevalence of diabetes in the rural population of Tessekere (Senegal) and investigate associated risk factors.

METHODS

Data from a 2015 survey of 500 individuals age 20 and over representative of the population of the municipality of Tessekere were used. Sociodemographic characteristics, health related variables, capillary whole blood glucose, and weight and height measurements of individuals were collected during face-to-face interviews. Statistical analyses used were bivariate tests and binary logistic regressions.

RESULTS

The percentage of individuals having impaired fasting glucose (IFG) is 6.6%. Those with fasting blood glucose (FBG) levels $\geqslant$ 126 mg/dL and/or currently being treated for diabetes is 4.2%. Only mean body mass index (BMI) is significantly higher among diabetic individuals and among those having FBG levels $\geqslant$ 110 mg/dL. After adjustment for sex, age, educational level, BMI and hypertension, only BMI

is associated with diabetes.

CONCLUSION

Prevalence of diabetes and IFG in our study correspond to the high range of rural sub-Saharan Africa prevalence. Diabetes is thus becoming a pressing public health concern, even in rural areas. But the risk factors identified in Tessekere suggest that the diabetes epidemic is still in the early stages, such that concerted action would make it possible to contain the devastating impact of this chronic condition.

Key words: Anthropology; Epidemiology; Sub-Saharan Africa; Diabetes

Core tip: Our study is one of the first, to our knowledge, to estimate the prevalence of diabetes in a rural Senegalese area. In the Tessekere municipality, diabetes prevalence is 4.2%, and that of impaired fasting glucose is 6.6%, corresponding to the high range of prevalence observed in rural sub-Saharan Africa. In our population study, emerging risk factors such as depression and material well-being (identified mainly in developed countries) are not associated with diabetes, indicating that this epidemic is in the early stages in this region.

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INTRODUCTION

The World Health Organization[1] estimates that globally, high blood glucose is the third highest risk factor for premature mortality. According to the International Diabetes Federation[2], some 415 million people worldwide are estimated to have diabetes and about 75% live in low- and middle-income countries. The prevalence of diabetes is increasing rapidly, and it is expected that by 2040 there will be 34.2 million adults in sub-Saharan Africa living with diabetes, more than double the number in 2015[2]. This increasing rate of diabetes mellitus is an additional burden to a region that continues to bear the brunt of communicable diseases such as tuberculosis and malaria[3]. Moreover, Africa Region has the highest proportion of undiagnosed diabetes; over two-thirds (66.7%) of people with diabetes are unaware they have the disease[2]. The diabetic condition is usually only diagnosed once patients are overtly symptomatic or present complications[4], which leads to increased risks of serious and fatal consequences associated with the progression of the disease.

Diabetes was virtually non-existent in West African populations about three decades ago[5,6]. But today, an estimated 4% of urban West African adults have diabetes[7], and this figure is higher in some countries: 7.7% in Ghana[8], 4.2% in Kenya[9], 4% and 7.7% in rural and urban Guinea respectively[10], and 8.8% in Nigeria[11] for example. Among non-modifiable risk factors, age is one of the most important, making the ageing of the sub-Saharan population a major determinant of the global rise in diabetes on the continent[12]. Gender trends, another non-modifiable risk factor, are not clear in Africa[6,7,12]. Genetic susceptibility, family history of diabetes and intrauterine influence are also classified as non-modifiable risk factors for diabetes[12].

Urbanization is known as a major modifiable risk factor for diabetes: Abubakari *et al*[7] have shown that individuals living in urban areas were over five times more likely to have diabetes than their rural counterparts. The higher diabetes prevalence in urban compared to rural settings is attributable to nutritional and lifestyle changes[12]. Urbanization is then associated with physical inactivity and adiposity, another modifiable risk factors for diabetes. Indeed, several studies have reported the independent association of higher adiposity with diabetes in sub-Saharan Africa[6,9,10,12-14]. Finally, Peer *et al*[12] have also mentioned that psychosocial stress or depressive syndrome might be considered a potential risk factor for diabetes.

Whereas Senegal is in the top five sub-Saharan African countries in terms of advanced nutritional transition status and dietary composition - which indicates increased risk for non-communicable diseases[15] - few studies have attempted to describe national trends in diabetes. In 1960, Payet *et al*[16] estimated the prevalence of diabetes in Dakar to be 1.1%. In 2015, the International Diabetes Federation reckoned the prevalence to be 1.8%[2]. In 2009 in Dakar, 17.8% of the population had a fasting blood glucose (FBG) level $\geqslant$ 110 mg/dL[17]. Information about diabetes prevalence in Senegalese rural areas is scarce. Consequently, the aim of this study is to estimate the prevalence of impaired fasting glucose (IFG) and diabetes in the rural population of Tessekere municipality (Senegal) and to investigate associated risk factors. Tessekere is a rural area populated mainly by nomadic pastoralists, whose culture and economy revolve around cattle. Our two hypotheses are therefore that the intense physical activity, low-fat diets and traditional way of life characterizing Ferlo's Fulani population would protect them from diabetes, leading to a low prevalence of this disease, and that considering the type of population, the diabetes epidemic is likely to be in the early stages. Emerging risk factors such as depression or stress, identified mainly in developed countries[18,19], will probably not be associated with diabetes in our population.

MATERIALS AND METHODS

Population sample

In order to carry out this study, a comprehensive

survey was conducted from February to August 2015 in the municipality of Tessekere (Ferlo region, northern Senegal). In 2014, according to Senegal's National Agency for Statistics and Demography (ANSD), a total of 8999 individuals aged 20 and over were living in Tessekere municipality[20]. The population sample selected for this study comprised 500 individuals aged 20 and over. The sample was constructed using the combined quota method (cross-section by age and gender) to strive for representativeness of the population of Tessekere aged 20 and over. Data from the ANSD dating from the last census (2013) were used. The quota variables used were gender (male/female) and age (20-29/30-39/40-49/50 and over).

Eight trained investigators (PhD students in Sociology, Medicine and Pharmacy) started out from different points each day to interview individuals in Wolof or Haalpulaar in each camp. Investigators had a certain number of individuals to interview to meet the quotas. Only one person was selected as a respondent in each home. Investigators went to the house, inquired about the inhabitants and then chose the first person they saw who met the characteristics needed for the quotas. In-person interviews were conducted. They ranged from 30 to 45 min, depending on respondent availability and desire to talk. As the objectives of this study include analysis with BMI, pregnant women were withdrawn from the sample, resulting in a sample of 496 individuals.

Variables studied

The socioeconomic and demographic variables collected were age (20-29/30-39/40-49/50 and over), gender (male/female) and educational level - defined in accordance with the educational system in Senegal - (0/1-5/6-9/10-12/over 12 years of school).

The following question was used as an indicator of economic conditions: "Given your household income, do you feel you … (1) live well? (2) live okay? (3) live okay, but you have to be careful? and (4) have difficulty making ends meet"? This question, taken directly from Razafindrakoto and Roubaud's study[21], has demonstrated validity and relevance in eight African capitals, including Dakar, to measure economic conditions in the context of subjective well-being. For the analyses, the answers were coded from 1 (poor) to 4 (prosperous). The Mini International Neuropsychiatric Interview (MINI) was used to diagnose major depressive episodes at the time of the interview[22,23].

The biological health variables collected were dysglycemia, blood pressure (BP), body mass index (BMI), waist circumference (WC) and waist-to-hip ratio (WHR). For dysglycemia, subjects were examined during the morning after fasting since the previous evening meal. The day before the investigation, subjects were informed of the need to have nothing to drink or eat in order to measure capillary whole blood glucose. Capillary whole blood (glucose) was obtained from a finger prick and was immediately analyzed using a Hemocue blood glucose analyzer®. The participants were then divided into three categories according to international standards[24]: Fasting plasma glucose < 110 mg/dL; IFG: Fasting plasma glucose levels between 110 and 125 mg/dL; those with diabetes, who had either been previously diagnosed diabetics or had capillary whole blood glucose value greater than or equal to 126 mg/dL.

In order to measure BP, we used an OMRON M5-I digital automatic blood pressure monitor (OMRON®, s'Hertogenbosch, The Netherlands). Measurements were taken on the upper right arm using an appropriate sized cuff while the participant was sitting and had rested for 5 min. Three readings were taken during the interview. The first was discarded, and the mean of the last two readings were used in the analysis. Hypertension (HTA) was defined as a systolic BP $\geqslant$ 140 mmHg (SBP) and/or a diastolic BP $\geqslant$ 90 mmHg (DBP) or reported treatment for hypertension.

Finally, overweight was defined as 25 $\leqslant$ BMI < 30; obesity corresponded to a BMI of $\geqslant$ 30; underweight to a BMI of < 18.5. WC of $\geqslant$ 102 cm in men and of $\geqslant$ 88 cm in women was considered central obesity. Lastly, a WHR of > 0.9 in men and a WHR of > 0.8 in women were considered central obesity[25].

Statistical analysis

Files compiled on the basis of the 496 questionnaires were processed and coded in Excel (2013). We used χ^2 tests to measure the presence, strength, and independence of statistical association of socio-demographic and biological variables and diabetes. We also carried out binary logistic regression analyses to estimate the risk factors for diabetes. All analyses were performed using SPSS software, version 20. A two-sided *P*-value of less than 0.05 was considered significant.

RESULTS

In our sample, more than two-thirds of the participants were aged < 40 years. Almost three-quarters had not attended school and the majority was not depressive. Proportions of men and women were balanced, as the proportions of people with good and poor material well-being. Most individuals were not hypertensive and not obese by WC, whereas 40% of individuals had central obesity measured by WHR. Prevalence of diabetes and IFG in our sample was 4.2% (95%CI: 2.44%-5.96%) and 6.6% (95%CI: 4.42%-8.78%) respectively.

Table 1 shows that in Tessekere, only mean BMI is significantly higher among diabetic individuals and among those having FBG $\geqslant$ 110 mg/dL. Furthermore, IFG is significantly more prevalent in individuals aged 50 and over and in individuals with central obesity (by WC and WHR).

The previously identified relationship between FBG, sociodemographic and biological health variables was

Table 1 Fasting blood glucose levels by sex, age, education, material well-being, Mini International Neuropsychiatric Interview, hypertension, waist circumference, waist-to-hip ratio, body mass index, systolic blood pressure and diastolic blood pressure in Dakar (n = 500)

Variables	Categories	< 126 mg/dL	⩾ 126 mg/dL	Test	< 110 mg/dL	⩾ 110 mg/dL	Test	Total
Total		475	21		463	33		496
Sex	Men	233	8	χ^2 = 0.967;	228	13	χ^2 = 1.196;	241
	Women	242	13	P = 0.326	235	20	P = 0.274	255
Education	0	358	15	χ^2 = 0.204;	350	23	χ^2 = 3.044;	373
level	1-5	82	4	P = 0.903	81	5	P = 0.218	86
	6-9	18	0		16	2		18
	10-12	11	2		10	3		13
	> 12	6	0		6	0		6
Age	20-29	193	7	χ^2 = 3.026;	189	11	χ^2 = 11.893;	200
bracket	30-39	112	3	P = 0.553	111	4	P = 0.018	115
	40-49	73	4		72	5		77
	⩾ 50	97	7		91	13		104
Material	Well	71	1	χ^2 = 5.554;	69	3	χ^2 = 2.351;	72
well-being	Okay	145	11	P = 0.135	142	14	P = 0.503	156
	Okay but careful	203	6		197	12		209
	Difficulties	56	3		55	4		59
MINI	Not depressive	438	20	χ^2 = 0.261;	426	32	χ^2 = 1.072;	458
	Depressive	37	1	P = 0.610	37	1	P = 0.301	38
HTA	HTA -	327	13	χ^2 = 0.449;	319	21	χ^2 = 0.396;	340
	HTA +	148	8	P = 0.503	144	12	P = 0.529	156
WC	Non obese	421	16	χ^2 = 2.970;	412	25	χ^2 = 5.143;	437
	Obese	54	5	P = 0.085	51	8	P = 0.023	59
WHR	Non obese	271	8	χ^2 = 2.937;	266	13	χ^2 = 4.082;	279
	Obese	204	13	P = 0.087	197	20	P = 0.043	217
BMI	Mean	20.8396	23.8	t = 3.303; P = 0.001	20.8	23.1	t = 3.155; P = 0.002	20.9
SBP	Mean	125.3	127.8	t = 0.400; P = 0.693	125.2	127.5	t = 0.470; P = 0.641	125.4
DBP	Mean	82.1	80.6	t = 0.579; P = 0.563	82.1	81.5	t = 0.301; P = 0.763	82.1

Education levels > 5 years have been aggregated in order to keep sufficient numbers for statistical tests. MINI: Mini International Neuropsychiatric Interview; HTA: Hypertension; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; WC: Waist circumference; WHR: Waist-to-hip ratio.

tested by binary logistic regression. The results of the binary logistic regression are presented in Table 2.

Results show that actually only BMI was associated with FBG ⩾ 126 mg/dL. Indeed, increased BMI is associated with increased risks of diabetes (Table 2). Furthermore, age and BMI were independently associated with IFG. Variables concerning central obesity (WC and WHR) were no longer associated with FBG after adjustment for age, sex and education level.

Finally, among individuals with FBG ⩾ 126 mg/dL, 5 (23.8%) were aware of their diabetic condition, 2 (9.5%) of the diabetics were treated, and 0 diabetic individuals under treatment had controlled FBG (*i.e.*, FBG < 126 mg/dL) (Figure 1).

DISCUSSION

The present study is to our knowledge one of the first to evaluate the prevalence of diabetes and IFG in a rural area in Senegal. The prevalence of diabetes in our sample is 4.2%, and that of IFG, 6.6%, corresponding to the high range of prevalence observed in rural sub-Saharan Africa, ranging from 0.8% to 6% for diabetes[26,27]. Our results thus confirm those obtained in Guinea[10], Nigeria[28] and Mali[29], suggesting that Fulani populations have a high prevalence of diabetes. The reasons for this high prevalence remain unclear. In the Tessekere municipality, Barral *et al*[30] showed that in 1981, a decreased consumption of cereals and an increased consumption of sugar (with tea, which was introduced recently at that time) and fat was observed since the 1950s, inducing a physiological modification that may have an impact on diabetes or hypertension. Indeed, following the severe droughts in the Sahelian region in the 1970s, local products (millet, leaves, milk, cowpea, *etc.*), which provided food security were replaced by imported foodstuffs (rice, peanut oil, pasta, bread, *etc.*) and by small ruminant holdings (sheep, goats) to compensate for economic losses. It is more than likely that these rapid changes in diet have gradually led to an increase in diabetes over the past 30 years.

The risk factors identified in our population do not differ from those of other countries[12]. Age and BMI are significant predictors of FBG ⩾ 110 mg/dL, but it is noteworthy that only BMI, and not age, is a significant predictor of diabetes in Tessekere. Indeed, it might be associated with two facts. First, in our study, the Fulani population presents a very high proportion of undiagnosed diabetes (76.19%). This result is in line with the results generally obtained in developing countries, where generally half to three-quarters of all cases are undetected[2,12]. But it also means that the condition is usually only diagnosed once patients

Table 2 Odds ratios for fasting blood glucose ⩾ 110 mg/dL and fasting blood glucose ⩾ 126 mg/dL by sex, age, education level and body mass index in Tessekere (n = 500)[a]

Variables	Categories	IFG					Diabetes				
		P	Odds ratios	IC for OR (95%)			*P*	Odds ratios	IC for OR (95%)		
Sex (men)	Women	0.744	1.153	0.49	-	2.716	0.805	1.141	0.4	-	3.254
Age bracket (⩾ 50 yr)[1]	< 50 yr	0.0271	2.731	1.12	-	6.663	0.228	2.006	0.647	-	6.216
Education level (⩾ 1 yr)[1]	0 yr	0.157	1.832	0.792	-	4.236	0.41	1.55	0.546	-	4.396
HTA (HTA-)	HTA +	0.449	0.717	0.304	-	1.695	0.673	0.797	0.277	-	2.289
BMI (continuous)		0.049[a]	1.095	1	-	1.198	0.018[a]	1.142	1.023	-	1.274
WHR (obese)	Not obese	0.334	1.532	0.645	-	3.635	0.396	1.577	0.551	-	4.508
WC (obese)	Not obese	0.927	1.053	0.348	-	3.181	0.688	0.756	0.193	-	2.966

[a]P < 0.05. [1]Education levels ⩾ 1 year and individuals < 50 years have been aggregated in order to keep sufficient numbers for statistical tests. HTA: Hypertension; WC: Waist circumference; WHR: Waist-to-hip ratio.

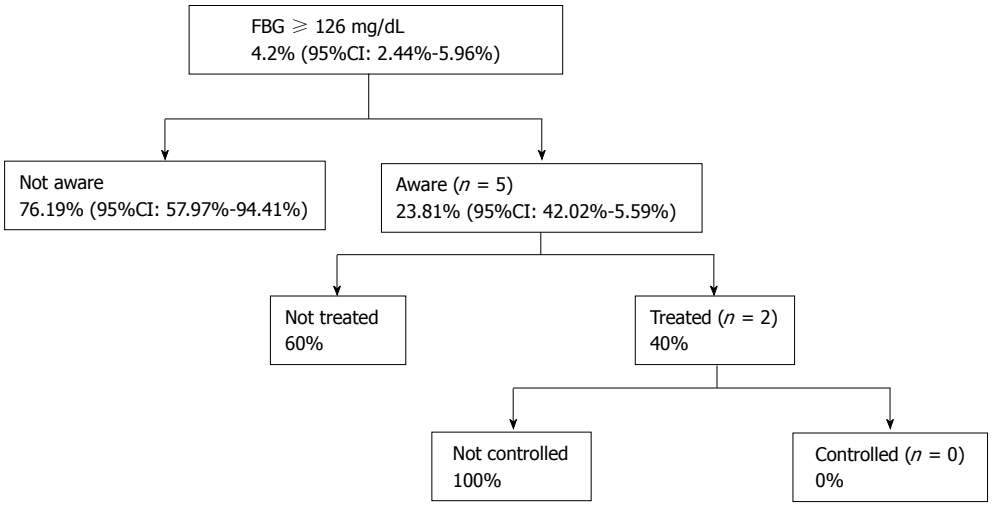

Figure 1 Prevalence of fasting blood glucose ⩾ 126 mg/dL, awareness among fasting blood glucose ⩾ 126 mg/dL, treatment among the aware, and control among the treated in Tessekere municipality. FBG: Fasting blood glucose.

are overtly symptomatic or present complications[4]. Consequently, the mortality associated with diabetes must be higher among older age groups, which explains the lack of correlation between age and the prevalence of diabetes. Secondly, even if Senegal is one of the five African countries with the most advanced nutrition transition[15], the Fulani population is very isolated (health centers, roads and stores located more than 5 km from camps, without motorized vehicles[31]), one of the poorest in Senegal, and seems to be just at the beginning of demographic, epidemiological and nutritional transitions. These characteristics could explain the lack of relationship between diabetes and age, as the diabetes epidemic appears to be nascent in our population. The same cause could also explain the lack of association between IFG or diabetes and central obesity. Indeed, whereas central obesity[14], depression[18] and material well-being[19] are generally identified risk factors in populations having long experienced diabetes, the recentness of the diabetes epidemic in our population may explain the absence of these risk factors.

Finally, the 4.2% prevalence of diabetes found in our study is comparable to the 4.6% observed in rural Senegal by Seck *et al*[32] but is far below the 8.5% to 12.9% prevalence reported for all other parts of the world[2]. In addition, the prevalence of IFG in our Tessekere sample is significantly lower than the 17.9%[17] or the 10.3%[10] observed respectively in Dakar and in urban Ghana with the same criteria and methods, which make direct comparison possible. These results are in line with the majority of the results concerning the urban-rural distribution of diabetes in sub-Saharan Africa[9,12]. Currently, 38% of the population of sub-Saharan Africa live in urban areas. But this proportion is predicted to increase to 45% by 2030, with a demographic inflection point to be attained by 2040: More urban than rural residents[33]. Due to inherent growth in urban districts and massive migration from rural areas[6], diabetes will certainly rise in the next few years in Senegal, where a focus on actions to optimize lifestyle management is critically important, given that obtaining drugs to treat diabetes is challenging due to cost and availability[34,35].

Our study has some limitations. First, as in many surveys, our FBG levels were based on capillary blood

measurements, which may have underestimated the prevalence rates[36]: The 2006 WHO/IDF update states that glycaemic values on venous and capillary plasma are identical but that plasma measures are 11% higher than whole blood measures. Second, HbA1c measurements are missing, but would have been important to accurately diagnose diabetes mellitus in our sample. Furthermore, the small absolute number of diabetic subjects makes assessment of the relationship to other variables examined difficult, and it is possible that only gross differences, such as the relation between diabetes and BMI will be found. Third, the small absolute number of diabetic subjects with known, treated and controlled diabetes made it impossible to perform trend analysis of age/sex subgroups. Finally, diagnosis of diabetes should not be based on a single abnormal result in an asymptomatic subject, so that future studies on the area should repeat the capillary whole blood glucose test for every subject.

The prevalence of diabetes in the rural area of Tessekere (Senegal) is 4.2%, and of particular concern is the high burden of undiagnosed and uncontrolled diabetes. If the nutritional transition is to develop in this part of the country, diabetes and its complications are certain to become a major health issue within several years. The challenge of diabetes in rural areas of sub-Saharan Africa is to provide accessible, affordable and optimal care for the management of the disease. It is then possible that collaboration with traditional healers would be appropriate and respectful of the populations' cultural values, and could represent a first step toward an integrative approach combining biomedical knowledge and traditional medicine.

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COMMENTS

Background

The World Health Organization estimates that globally, high blood glucose is the third highest risk factor for premature mortality. The increased rising rate of diabetes mellitus is an additional burden to sub-Saharan Africa that continues to bear the brunt of communicable diseases such as tuberculosis and malaria. Moreover, Africa Region has the highest proportion of undiagnosed diabetes; over two-thirds (66.7%) of people with diabetes are unaware they have the disease. Whereas Senegal is in the top five sub-Saharan African countries in terms of advanced nutritional transition status and dietary composition - which indicates increased risk for non-communicable diseases - few studies have attempted to describe national trends in diabetes and no information exists about diabetes prevalence in rural Senegalese areas.

Research frontiers

Diabetes was virtually non-existent in West African populations about three decades ago. But today, it is estimated that 4% of urban West African adults have diabetes, and this figure is higher in some countries: 7.7% in Ghana, 4.2% in Kenya, 4% and 7.7% in rural and urban Guinea respectively, 8.8% in Nigeria for example. Identified risk factors for diabetes in sub-Saharan Africa are age, family history of diabetes and intrauterine influence. Urbanization is known as a major modifiable risk factor for diabetes, attributable to nutritional and lifestyle changes and physical inactivity. Finally, in developed countries, psychosocial stress or depressive syndrome might be considered potential risk factors for diabetes.

Innovations and breakthroughs

The study is the first, to our knowledge, to estimate the prevalence of diabetes in a rural Senegalese area. In Tessekere municipality, diabetes prevalence is 4.2%, and that of impaired fasting glucose, 6.6%, corresponding to the high range of prevalence observed in rural sub-Saharan Africa. In the population study, emerging risk factors such as depression and material well-being (identified mainly in developed countries) are not associated with diabetes, indicating that this epidemic is in the early stages in this region.

Applications

It seems necessary to study the determinants of the high prevalence of diabetes observed in Fulani in sub-Saharan Africa. The study also casts doubt on the relationship between emerging factors for diabetes such as depression and material well-being and stages of the nutritional transition.

Peer-review

The introduction provides sufficient background and includes all relevant references. The research design is appropriate. The methods are adequately described. The results are clearly presented. The conclusions are supported by the results.

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ORIGINAL ARTICLE

Observational Study

Quality of sleep and its determinants among people with type 2 diabetes mellitus in Northwest of Iran

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Abstract

AIM

To examine sleep quality and its determinants among people with type 2 diabetes mellitus (T2DM).

METHODS

This is a cross-sectional study conducted among diabetic patients referring to Ardabil diabetes clinic in Northwest of Iran. Information on sleep quality was collected using Pittsburg Sleep Quality Index (PSQI). A questionnaire was used to collect data on sociodemographic lifestyle factors and psychological distress. This questionnaire was completed through an interview, and clinical information was extracted from patient's record. Data analysis was done using SPSS software version 23 and univariate and

multivariate analyses.

RESULTS

Study participants consist of 256 people with T2DM the majority of whom were women (70%), and mean age of participants was 54.06 ± 9.09. The mean of total score of PSQI was 5.56 ± 3.34. Relative to younger age group, the middle-aged people with T2DM were twice more likely to be poor sleeper; the adjusted OR was 2.03 (95%CI: 1.01-4.08); and those with longer duration of diabetes were about 1.8 times more likely to report poor quality of sleep (ORadj = 1.77, 95%CI: 0.98-3.13). Participants with cholesterol level ⩾ 240 mg/dL were about twice more likely to be poor sleeper (ORadj = 1.99, 95%CI: 1.01-3.94). The odds of being poor sleeper increased as the level of distress increased (1.84-4.09).

CONCLUSION

As indicated by the results of the present study, some factors including age, duration of disease, psychological distress and high level of cholesterol were independently associated with poor sleep quality.

Key words: Type 2 diabetes mellitus; Lifestyle; Pittsburg Sleep Quality Index; Hypercholesterolemia; Psychological distress

Core tip: In Iran, diabetes is one of the health concerns with a prevalence of 8.5% because of lifestyle changes following rapid urbanization. Few studies have been conducted in Iran concerning quality of sleep among people with type 2 diabetes mellitus. Therefore, the present study aimed to examine the quality of sleep among people with type-2 diabetes referring to diabetes clinic in Ardabil, Northwest of Iran where diabetes is a health research priority in this province. The results showed that, age, duration of disease, psychological distress and high level of cholesterol were independently associated with poor sleep quality. Therefore, promotion of diabetes management, regular primary care and psychological consultation are recommended in order to improve sleep quality among people with type 2 diabetes.

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INTRODUCTION

Diabetes is one of the most important health concerns in societies[1], and according to estimates by the International Federation of Diabetes (IDF), there were 415 million adults with diabetes around the globe in 2015, even increasing in the following years[2].

Sleep disorder as a new risk factor for diabetes plays an important role in diabetes occurrence through neuro-metabolic pathway[3-5]. Cortisol level increases following sleep deprivation which inhibits insulin production and may induce pre-diabetic or diabetic states in long term. In addition, insulin sensitivity is reduced following the sleep disorder, and impairment in sleep quality or quantity is consequently followed by blood glucose elevation affecting the process of development of diabetes[6]. It has been shown that reduction in duration of night sleep to four h over six successive nights can cause Impaired Glucose Tolerance (IGT) in adults[7]. In people with diabetes, a balance between insulin secretion and glucose uptake is impaired; therefore, following sleep deprivation due to high level of cortisol and reduced glucose metabolism, glucose levels is increased causing diabetes aggravation[8].

A range of sleep disorders are common among people with T2DM, including sleep apnea, insomnia, periodic limb movements, circadian rhythm, sleep hygiene and psychoactive drug use, which, in sleep apnea, is the most common reported disorder[9,10].

There is evidence that approximately one third of people with diabetes suffered from sleep problems whilst it was only 8.2% in control group without DM[11]. In another study, more than half of the people with T2DM were "poor sleepers"[12]. It has also been shown that using the Pittsburg Sleep Quality Index (PSQI) as the validated tool for measuring quality of sleep[9], lower score of PSQI were reported by people with type 2 diabetes[13]. Evidence showed that poor sleep quality among people with T2DM is associated with longer duration of diabetes, poor glycemic control (hemoglobin A1c > 7%), normal body mass index, and hypertension[13]. Studies also reported that the high prevalence of poor sleep quality among people with T2DM has a negative impact on glycemic control[14,15]. A recent systematic review and meta-analysis revealed that poor quantity and quality of sleep were associated with an increased HbA1C[16]. Therefore, sleep quality improvement plays an important role in glycemic control among people with T2DM. A good sleep quality should be considered as an important component in the prevention and management of T2DM.

In Iran, diabetes is one of the health concerns with a prevalence of 8.5% due to lifestyle changes following rapid urbanization[2]. Few studies have been conducted in Iran concerning quality of sleep among people with T2DM[17,18]. We could not find any published paper in this regard in northwest of Iran; therefore, the present study aimed at examining the quality of sleep among people with type-2 diabetes referring to diabetes clinic in Ardabil, Northwest of Iran where diabetes is a health research priority[19], and inadequate quality of care has been reported in some regional studies and literature[20,21]. We aimed at assessing a range of

different socioeconomic and diabetes care factors as well as psychological distress regarding sleep quality among people with T2DM.

MATERIALS AND METHODS

The present study is a cross-sectional study conducted between June 2013 and March 2014 consisting of 256 type-2 diabetic patients referring to diabetes clinic of Ardabil. This clinic is located in Imam Khomeini Hospital in Ardebil, Northwest of Iran providing specialty and subspecialty services for diabetic patients referring from the neighboring towns and villages of Ardabil province. Inclusion criteria were possession of healthcare profile in the clinic, diagnosed with type Ⅱ diabetes and being in the age range of 20-70 years. Demographic information and the characteristics of the disease, treatment and the care measure variables such as body mass index (BMI), blood pressure, cholesterol and HbA_1C were collected. Information on sleep quality was obtained using PSQI[22]. This questionnaire evaluates 7 aspects of sleep quality including sleep quality, delay in falling asleep, sleep duration, normal quality of sleep, sleep disturbance, use of sleep medications and dysfunction during the day. The score range of the questionnaire is between 0 and 21, and a score of above 5 is considered as sleep disorder (higher scores indicate higher level of sleep disorder). The Persian version of the questionnaire is composed of 9 main questions; questions 1-4 are related to sleep and waking h and are responded quantitatively and questions 5-9 are scored using a 0-3 Likert scale. Question 5 has 10 subsets investigating problems related to sleep over the past month. The reliability and validity of the Persian version of this questionnaire are confirmed[23]. Patients were classified into two groups according to PSQI: Poor-sleeper group (PQSI > 5) and good-sleeper group (PQSI $\leqslant$ 5)[22]. Psychological distress was assessed using the Kessler's Psychological Distress Scale (K10). Questions are scored using a five likert scale; all of the time, most of the time, some of the time, a little of the time and none of the time. The maximum score is 50 indicating severe distress; the minimum score is 10 indicating no distress[24]. Patients were divided into 4 groups according to K10 score; no distress (< 20), mild (20-24), moderate (25-29), and severe (30 and higher). The reliability and validity of the Persian version of this questionnaire has been confirmed[25].

Questionnaires were completed by two trained staff in the diabetes clinic of Ardabil *via* a face-to-face interview. The study data were analyzed using descriptive and analytical statistical techniques through SPSS software version 23. Univariate and multivariate logistic regression models with classified PSQI score as poor (> 5) *vs* good ($\leqslant$ 5) sleepers were used as dependent variable to estimate crude and adjusted odds ratios (ORs) with 95% confidence intervals (CI). Adjustment was performed for gender, age, diabetes duration, treatment options, complications, HbA_1C, cholesterol level, hypertension, BMI, and psychological distress (K10). Significance level was set at 0.05 (P < 0.05).

Table 1 Demographic characteristics of the patients with type-2 diabetes in diabetes clinic of Ardabil (n = 256) in 2014

Characteristics	n (%)
Age group (yr)	
< 50	68 (26.6)
50-59	113 (44.1)
> 60	75 (29.3)
Sex	
Male	75 (29.3)
Female	181 (70.7)
Education	
Illiterate	127 (49.6)
Primary school	70 (27.3)
Secondary school and higher	59 (23)
Income	
Low (< 500)	151 (71.6)
Acceptable	60 (28.4)
Marital status	
Married	227 (88.7)
Single/divorced/widow	29 (11.3)
Occupation	
In-paid work	41 (16)
Not working	215 (84)
Smoking	
Yes	232 (92.4)
No	19 (7.6)
Body mass index	
< 25	49 (19.1)
25-29.9	101 (39.5)
⩾ 30	106 (41.4)

This study is a part of a diabetes care project approved by the Medical Ethics Committee of Tabriz University of Medical Sciences (Ethic Number: TBZ-MED.1392.5.4.7580). Moreover, a written consent was obtained from all of the participants.

RESULTS

The mean age of the patients was 54.06 ± 9.09, and most of the patients were women (71%). The majority were married living with their spouse at the time of the research (89%). About 50% of the participants were illiterate and, only 3.5% had a university degree. The majority had low income level (72%), and only 16% were engaged in in-paid work. 8% of study population were smokers, and only 19% had normal weight (Table 1).

The patients' mean of real sleep h was 6.71 ± 1.45. In response to the question "During the past month, how would you rate your sleep quality overall?" 15.7% of the patients reported their sleep quality good, 61% relatively good, 19.3% relatively bad, and 3.9% very bad. Moreover, about 23.1% of the patients reported their real sleep h below 5 h, and 10.4% more than 9 h.

In univariate analysis, people in age group 50-59 were more frequently reported to be poor sleepers followed by those in age group 60 and over; the

Table 2 Quality of sleep among diabetic patients by socio-demographic and clinical factors, 2014-2015, Northwest of Iran *n* (%)

Characteristics	Total	PQSI ⩽ 5 (good sleepers)	PQSI > 5 (poor sleepers)	*P* value[1]
Age group (yr)				0.136
< 50	68	48 (70.6)	20 (29.4)	
50-59	113	63 (55.8)	50 (44.2)	
⩾ 60	75	47 (62.7)	28 (37.3)	
Sex				0.294
Male	75	50 (66.7)	25 (33.3)	
Female	181	108 (59.7)	73 (40.3)	
Education				0.518
Illiterate	127	74 (58.3)	53 (41.7)	
Primary school	70	45 (64.3)	25 (35.7)	
Secondary school and higher	59	39 (66.1)	20 (33.9)	
Income				0.662
Low (< 500)	151	93 (61.6)	58 (38.4)	
Acceptable	60	35 (58.3)	25 (41.7)	
Marital status				0.96
Married	227	136 (59.9)	91 (40.1)	
Single/divorced/ widow	29	22 (68.2)	7 (31.8)	
Occupation				0.552
In-paid work	41	27 (65.9)	14 (34.1)	
Not working	215	131 (60.1)	84 (39.1)	
Duration of disease (yr)				0.003
< 6	114	82 (71.9)	32 (28.1)	
⩾ 6	140	75 (53.6)	65 (46.4)	
Complications				0.013
No	134	92 (68.7)	42 (31.3)	
Yes	118	63 (63.4)	55 (46.6)	
Smoking				0.18
Yes	19	9 (47.4)	10 (52.6)	
No	232	146 (62.9)	86 (37.1)	
BMI				0.84
< 25	49	32 (65.3)	17 (34.7)	
25-29.9	101	61 (59.4)	40 (39.6)	
⩾ 30	106	65 (61.3)	41 (38.7)	
HbA1C				0.882
< 7	91	57 (62.6)	34 (37.4)	
⩾ 7	165	101 (61.2)	64 (38.8)	
Hypertension				0.089
Controlled	81	56 (69.1)	25 (30.9)	
Uncontrolled	149	86 (57.7)	63 (42.3)	
Total cholesterol				0.016
Desirable	205	134 (65.4)	71 (34.6)	
⩾ 240	51	24 (47.1)	27 (52.9)	
Psychological distress (K10)				< 0.001
No	88	67 (76.1)	21 (23.9)	
Mild	85	54 (63.5)	31 (36.5)	
Moderate	46	22 (47.8)	24 (52.2)	
Severe	37	15 (40.5)	22 (59.5)	
Treatment option				0.584
Oral medication	149	91 (61.1)	58 (38.9)	
Insulin + oral OR	74	44 (59.5)	30 (40.5)	
Insulin				
Other	33	23 (69.7)	10 (30.3)	

[1]*P* value was reported based on univariate and multivariate logistic regression tests.

same was true for women than men; however, the associations were not statistically significant. Poor sleep quality decreased as education level increased. In other words, the number of poor sleepers was the highest among illiterate study participants. Poor sleep was more common in married, low income and not-working study population (Table 2). In regard with lifestyle and diabetes care measures, people with diabetes with longer duration of disease (> 6 years), complications, and those with high level of cholesterol were more likely to report poor sleep quality. Hypertension, poor glycemic control (HbA1C level ⩾ 7), BMI, smoking and treatment option were not significantly associated with sleep quality. Study participants with higher score of psychological distress were more likely to be poor sleepers compared to those with no distress. Poor sleep quality increased as the score of psychological distress increased (*P* value < 0.001).

In multivariate logistic regression model, only duration of disease, age, cholesterol level, and psychological distress were remained as independent predictors of sleep quality (Table 3). Relative to younger age group, the middle-aged people with T2DM were twice more likely to be poor sleeper. The adjusted OR was 2.03 (95%CI: 1.01-4.08) for age group 50-59 years. Those with longer duration of diabetes were about 1.8 times more likely to report poor quality of sleep than those with shorter period of disease (ORadj = 1.77, 95%CI: 0.98-3.13). Study participants with cholesterol level ⩾ 240 mg/dL were about twice more likely to be poor sleeper compared to those with desirable level of cholesterol (ORadj = 1.99, 95%CI: 1.01-3.94). The odds of being poor sleeper increased as the level of distress increased (1.84-4.09); with the highest level in those with severe psychological distress compared to those with no distress (ORadj = 4.09, 95%CI: 1.71-9.77).

DISCUSSION

The present study investigated the quality of sleep and its correlates among people with T2DM referring to diabetes clinic of Ardabil, Northwest of Iran. The results of the present study indicated that 38% of the patients were classified as poor sleeper (PSQI > 5) less than other studies reporting 46-71 percent of the patients with PSQI scores above 5[12,18,26,27].

In this study, the mean of PSQI total score was 5.56 ± 3.34 which was similar to those reported by Ghanei *et al*[18] (5.5 ± 4.4). However, it was lower than other studies which reported greater mean score of PSQI[18,27,28]. In this study, 66.5% of participants reported their adequate sleep between 6-8 h; similar findings were reported from other studies conducted in Iran[29]. Moreover, in the present study, 10.4% of the patients had real sleep duration of equal or more than 9 h, and about 21% of the patients reported their real sleep duration to be ⩽ 5 h higher than the other[7,30]. Based on a meta-analysis conducted in 2010, lack of sleep or insufficient sleep and long sleep duration were known as risk factors of T2DM[31]. According to the results of different studies,

Table 3 Predictors of sleep quality among diabetic patients (outcome poor sleeper *vs* good sleepers)

Characteristics	OR crude (95%CI)	OR adjusted (95%CI)
Age group (yr)		
< 50	Ref	Ref
50-59	1.905 (1.004-3.613)	2.033 (1.014-4.077)
⩾ 60	1.430 (0.709-2.881)	1.223 (0.561-2.668)
P value	0.139	0.097
Duration of disease (yr)		
< 6	--	--
⩾ 6	2.221 (1.312-3.760)	1.767 (0.981-3.182)
P value	0.003	0.058
Complications		
No	Ref	Ref
Yes	1.912 (1.144-3.197)	1.438 (0.805-2.568)
P value	0.013	0.219
Psychological distress (K10)		
No distress	Ref	Ref
Mild distress	1.832 (0.947-3.543)	1.835 (0.910-3.701)
Moderate distress	3.481 (1.631-7.429)	3.282 (1.430-7.533)
High distress	4.679 (2.063-10.615)	4.087 (1.710-9.765)
P value	< 0.001	0.005
Total cholesterol		
Desirable	Ref	Ref
⩾ 240	2.123 (1.142-3.949)	1.989 (1.006-3.935)
P value	0.017	0.048

P value was reported based on univariate and multivariate logistic regression tests. Only variables with significant association in either univariate or multivariate models have been reported. Dependent variable: Sleep quality; OR: Odds ratio.

risk of death from cardiovascular diseases and symptoms of diabetes increase among people who sleep less than 7 or more than 8 h a day[32].

Our results showed that only duration of disease, age, cholesterol level, and psychological distress remained as independent predictors of sleep quality. These findings were in agreement with the results of some studies[12,13]. In addition, similar to our results, another study found that diabetic people with poor sleep quality had higher total cholesterol compared to those with good sleep quality[29]. In this study, gender, hypertension, HbA_1C, complications, treatment options, and BMI were not significantly associated with sleep quality. In contrast, the results of a study carried out by Maracy *et al*[33] indicated that sleep quality among women was worse than that of men. The majority of study participants in our study were women; therefore, gender differences could not be fully assessed.

Unlike our study, a significant relationship between HbA_1C and PSQI has been reported while the association between the patients' fasting blood sugar (FBS), blood pressure, LDL, triglyceride, and BMI was not statistically significant[18,26,33]. A linear correlation between sleep duration and glycemic control among type 2 diabetics has been reported[34]. A recent study found a significant relationship between overweight/obesity = with sleep quality among people with diabetes[35].

Regarding the negative effects of treatment with insulin on diabetic patients and reduction of satisfaction among patients treated with insulin injection[36,37], in the present study, the percentage of poor sleeper was a bit higher among those treated through insulin injection alone or with oral medication (40% compared to 39% in oral medication group) and much higher than other treatment group, though the difference was not statistically significant. There are some studies that showed the associations between poor sleep quality and insulin therapy[12,26,38]. The current study found that the odds of being poor sleeper increased as the level of psychological distress increased from 1.84 to 4.09. There is evidence that depression is an independent predictor of poor sleep quality among people with T2DM in insulin therapy[38].

This study has some strengths and limitations. There are few studies in Iran assessing the sleep quality among people with T2DM; therefore, the results of this study can provide valuable information for clinicians in order to enhance the management of diabetes. We included a range of different variables especially psychological distress less assessed in previous studies. One limitation that could be mentioned in the present study is that the majority of study population were women (70%), so gender differences cannot be appropriately probed. The generalizability is another limitation of the present study since we recruited participants from diabetes clinic through the convenience sampling method. Although this clinic is a diabetes center, the people referring to this center might not be representative sample of entire population with diabetes. Finally, we did not collect information on caffeine intake, medications other than diabetes specific medications, and breathing disorder that might have effect on patient sleep.

In conclusion, according to the results of the present study, age, duration of disease, psychological distress and high level of cholesterol were independently associated with poor sleep quality. Therefore, promotion of diabetes management, regular primary care and psychological consultation are recommended in order to improve sleep quality among people with T2DM.

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COMMENTS

Background

Sleep disorder as a new risk factor for diabetes plays an important role in diabetes occurrence through neuro-metabolic pathway. Studies also reported that the high prevalence of poor sleep quality among people with type 2 diabetes mellitus (T2DM) has a negative impact on glycemic control. Research on sleep quality among diabetic people is scarce therefore, assessing a range of different socioeconomic and diabetes care factors as well as psychological

distress regarding sleep quality among people with T2DM is needed.

Research frontiers

Diabetes is one of the health concerns in Iran due to lifestyle changes following rapid urbanization and will continue to rise in the next decades. Ardabil; a Northwestern province of Iran is among provinces, in which diabetes is very common and low quality of life and diabetes care has been reported from this area. Quality of sleep as a related factor to diabetes occurrence and control has not been assessed in this province, therefore findings of this study can provide a clearer picture of the problem in order to implement an appropriate public health interventions.

Innovations and breakthroughs

Limited data is available on quality of life among people with diabetes in Iran and they could not find any study in Northwest of Iran where the diabetes is one of the heath research priorities in this area.

Applications

Considering the negative impact of poor quality of sleep on glycemic control among people with TD2M, identification of associated factors can contribute to promotion of diabetes management.

Terminology

Pittsburg Sleep Quality Index (PSQI): This is a valid questionnaire evaluates 7 aspects of sleep quality including sleep quality, delay in falling asleep, sleep duration, normal quality of sleep, sleep disturbance, use of sleep medications and dysfunction during the day. Kessler psychological distress (K10): A 10-item questionnaire intended to measure the level of distress based on questions about anxiety and depressive symptoms over the recent 4 wk.

Peer-review

Authors did a nice effort to address this important issue among Iranian population. While the results are expected, the data worth publication and will be of interest to diabetes patients.

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ORIGINAL ARTICLE

Prospective Study

Are body mass index and waist circumference significant predictors of diabetes and prediabetes risk: Results from a population based cohort study

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Abstract

AIM

To determine the predictive role of body mass index (BMI) and waist circumference (WC) for diabetes and prediabetes risk in future in total sample as well as in men and women separately.

METHODS

In a population based cohort study, 1765 with mean ± SD age: 42.32 ± 6.18 healthy participants were followed up from 2003 till 2013 (n = 960). Anthropometric and biochemical measures of participants were evaluated regularly during the follow up period. BMI and WC measures at baseline and diabetes and prediabetes status of participants at 2013 were determined. Multivariable logistic regression analysis was used for determining the risk of diabetes and prediabetes considering important potential confounding variables. Receiver operating

characteristic curve analysis was conducted to determine the best cut of values of BMI and WC for diabetes and prediabetes.

RESULTS

At 2013, among participants who had complete data, 45 and 307 people were diabetic and prediabetic, respectively. In final fully adjusted model, BMI value was a significant predictor of diabetes (RR = 1.39, 95%CI: 1.06-1.82 and AUC = 0.68, 95%CI: 0.59-0.75; $P < 0.001$) however not a significant risk factor for prediabetes. Also, WC was a significant predictor for diabetes (RR = 1.2, 95%CI: 1.05-1.38 and AUC = 0.67, 95%CI: 0.6-0.75) but not significant risk factor for prediabetes. Similar results were observed in both genders.

CONCLUSION

General and abdominal obesity are significant risk factors for diabetes in future.

Key words: Diabetes; Prediabetes; Waist circumference; Body mass index; Anthropometric measure

Core tip: The predictive powers of body mass index (BMI) and waist circumference (WC) were similar in predicting the incidence risk of diabetes in either gender. The cut-off points for predicting diabetes in men and women were different. Defined cut-off points based on maximum sensitivity plus specificity values suggested that in men, BMI of 26.2 kg/m^2 and WC of 89.7 cm, and in women, BMI of 28.6 kg/m^2 and WC of 84.3 cm would predict Isfahanian population at high risk for developing diabetes.

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INTRODUCTION

The increased prevalence of obesity in the world[1] is a major concern as it is strongly related to multiple metabolic disorders[2]. General obesity measured by body mass index (BMI) is a known risk factor for diabetes[2]. Although BMI is often advocated as a simple measure to determine disease risk, it has several limitations. First, lean mass and fat mass could not be differentiated for a given BMI across age, sex and race[3]. Second, fat distribution could not be distinguished by BMI[4,5], whilst it has been generally accepted that visceral adiposity plays more important role in developing insulin resistance and diabetes rather than overall adiposity[6-8]. Therefore, waist circumference (WC) was developed as an abdominal adiposity measure which considers fat distribution.

Although in most populations WC is a stronger predictor for diabetes compared with BMI[7,8], no significant differences were observed between WC and BMI in Japanese[9] and Iranians[10] to predict disease risk. In addition, available risk thresholds predominantly come from European populations which might not be applicable to the Asian population due to differences in genetics and obesity pattern. Therefore, it is essential to identify the best anthropometric index and effective risk thresholds for adiposity measures to develop appropriate preventive strategies in each population.

Based on International Diabetes Federation (IDF)'s recommendation, WC cut-off values for clinical practice should be determined in different ethnicities[11]. Although IDF has suggested WC greater than 90 and 80 cm, respectively for Asian men and women, as cut-off point for abdominal obesity, there is no consensus for WC cut-off point in Iranians. Studies in this regard have suggested that 89-95 cm for men and 85-97 cm for women may be optimal cut-off points for abdominal obesity[12-15]. Nevertheless, to the best of our knowledge, there is only one longitudinal study among Iranians which has determined cut-off point of WC for detecting cardiovascular disease risk[15], and other cut-off points come from cross-sectional surveys[12-14,16]. These values might be limited because of the design of study.

The present study aimed to prospectively determine the effective anthropometric measures to predict the risk of type 2 diabetes and prediabetes also estimate the optimal cut-off point of WC and BMI by following up non-diabetic participants at baseline examination. Estimated cut-off points by this study will contribute to detect individuals at higher risk of developing type 2 diabetes as well as prediabetes in the Iranian population.

MATERIALS AND METHODS

Study subjects

Subjects in the present study were the participants in the Isfahan Diabetes Prevention Study (IDPS), an ongoing cohort study in central Iran. The aim of this study is evaluating the role of diet and physical activity in the prevention or delay the developing of diabetes in first-degree relatives (FDRs) of patients with type 2 diabetes. This study was run between 2003 and 2013. One thousand, seven hundred and sixty-five healthy participants including 446 (25.3%) males and 1371 (74.6%) females were selected from a consecutive sample who attended in the clinics of Isfahan Endocrine and Metabolism Research Center. Data from 960 people including 255 (25.5%) male and 705 (73.4%) female at 2013 were subjected to statistical analysis. Health status and potential risk factors for diabetes were assessed using a questionnaire. To

update demographic, anthropometric, and lifestyle information as well as diagnosis new diabetic cases, follow-up tests were run according to a medical care standard in diabetes[17]. Accordingly, participants with impaired 2-h OGTT at baseline were annually tested, and individuals with normal 75 g 2-h oral glucose test tolerance (2-h OGTT) were tested at least at 3-year intervals. More details regarding the participants and methodology of IDPS have been described elsewhere[18]. Informed written consent was obtained from all study participants and the Ethical Committee of Isfahan University of Medical Sciences approved the protocol of study.

Anthropometric assessment

Anthropometric indices were measured by well-trained examiners at baseline while participants were minimally clothed and without footwear. Weight was measured using a balanced scale and recorded to the nearest 0.1 kg. Height was determined using a wall-fixed tape measure while participants were in a normal standing position and recorded to the nearest 0.5 cm. WC and hip circumference were determined using a metal tape measure without imposing any pressure to body surface and were recorded to the nearest 0.5 cm. The location for measuring WC was considered as the narrowest level between the lowest rib and iliac crest, whilst for hip circumference was conserved as the largest level[19]. BMI was calculated as body weight in kilogram divided by height in Square meter.

Laboratory measurement

Biochemical tests including lipid profile, fasting plasma glucose (FPG) and OGTT were carried out for all participants. To determine lipid profile and FPG, a blood sample was drawn from all participants after 10-12 h overnight fasting. Postprandial plasma glucose was measured using venous blood sample at 30, 60, and 120 min after oral glucose administration. Plasma glucose and lipid profile concentrations were determined using enzymatic colorimetric method (ParsAzmoon, Tehran, Iran) adapted to a Selectra-2 auto-analyzer (Vital Scientific, Spankeren, Netherlands). Serum concentration of low-density lipoprotein cholesterol (LDL-C) was calculated by Friedwald equation in individuals with serum TG levels < 400 mg/dL[20]. HbA1c concentrations were measured in whole blood samples *via* the pink reagent kit on a DS5 analyzer. Both intra- and inter-assay coefficients of variability (CVs) were < 2.2% for all markers.

Definition of diabetes

The criteria for the diagnosis of diabetes and impaired glucose tolerance test were based on the American diabetes association (ADA) definition. Accordingly, cut-off point for impaired fasting glucose was considered as 100 mg/dL[17]. Diabetes was defined as FPG $\geqslant$ 126 mg/dL, or HbA1C $\geqslant$ 6.5% or 2-h OGTT $\geqslant$ 200 mg/dL.

Assessment of other variables

Blood pressure was measured using a Mercury sphygmomanometer while subjects were in seated position two times with at least 30 s interval between measurements. The mean of two measurements was recorded as the subject's blood pressure. Hypertriglyceridemia was defined as serum TG $\geqslant$ 150 mg/dL, high LDL-C as LDL-C $\geqslant$ 130 mg/dL, hypercholesterolemia asTC $\geqslant$ 200 mg/dL and low HDL-C as HDL-C < 50 mg/dL in female and < 40 mg/dL in male. According to the JNC and WHO Guideline criteria, hypertension was defined as systolic blood pressure (SBP) $\geqslant$ 130 mmHg, diastolic blood pressure (DBP) $\geqslant$ 85 mmHg and/or antihypertensive medications[21].

Statistical analysis

Continuous and categorical data were presented as mean ± SD. Normality of quantitative data was evaluated using Kolmogorov-Smirnov test and Q-Q plot. Positive skewed data was subjected to logarithmic transformation. χ^2 test was used for evaluating the association between categorical data. Between groups comparisons of quantitative data were conducted using Analysis of variance (ANOVA) or nonparametric Kruskal-Wallis tests. To determine the association between BMI and WC values at baseline (2003) as an independent variable and type 2diabetes and prediabetes at 2013, we used binary logistic regression analysis in different models. In these analyses, after obtaining relative risk (RR) and 95% confidence interval (95%CI) in crude model, adjustment was made for age and sex, smoking, positive family history in the first model. Additional adjustment was made for physical activity and energy intake in the second model. In third model adjustment additionally was done for FBS and HbA1c. Finally, adjustment was made for all mentioned variables and lipid profile indices (including TG, LDL, HDL and cholesterol) and blood pressure.

The predictive values of BMI and WC values for type 2 diabetes and prediabetes were evaluated using receiver operating characteristic curve (ROC) analysis and area under the curve (AUC) and its 95%CI. The optimal sensitivity and specificity for different cut off values of BMI and WC were calculated using Youden index. Statistical analyses were performed using statistical package for social science (SPSS version 15, SPSS, Inc., IL, United States).

RESULTS

General characteristics of participants at baseline are presented in Table 1. Individuals who affected by diabetes after 10 years follow up had greater BMI, WC, hip circumference, waist to hip ratio, fasting blood sugar, glycemic response, total cholesterol, triglyceride and systolic and diastolic blood pressures at baseline. Abdominal obesity at baseline was more prevalent

Table 1 Characteristics of study population at baseline

	Normal (n = 599)[1]	Pre-diabetes (n = 307)[1]	Diabetes (n = 45)[1]	P value
Whole population				
Age (yr)	42.06 ± 6.17	42.72 ± 6.20	43.20 ± 6.19	0.197
Energy intake (kcal/d)	1844.32 ± 553.85	1793.43 ± 571.48	1908.95 ± 598.94	0.646
Weight (kg)	72.09 ± 12.10	73.51 ± 12.49	78.22 ± 11.96	0.943
Height (cm)	160.21 ± 8.31	160.09 ± 8.62	159.81 ± 8.58	0.197
Body mass index (kg/m^2)	28.09 ± 4.17	28.66 ± 4.18	30.63 ± 4.15	< 0.0001
Waist circumference (cm)	87.00 ± 9.58	88.72 ± 9.75	92.86 ± 9.08	< 0.0001
Hip circumference (cm)	106.21 ± 8.54	107.35 ± 8.77	110.35 ± 9.27	0.003
Waist to hip ratio	0.82 ± 0.07	0.83 ± 0.07	0.84 ± 0.05	0.078
Fasting blood sugar	87.04 ± 7.99	89.30 ± 7.03	91.0 ± 6.14	< 0.0001
Blood sugar after 30 min (mg/dL)	127.41 ± 25.19	136.43 ± 26.48	141.49 ± 25.48	< 0.0001
Blood sugar after 60 min (mg/dL)	123.02 ± 32.06	136.19 ± 31.17	151.81 ± 36.61	< 0.0001
Blood sugar after 120 min (mg/dL)	98.11 ± 21.15	104.81 ± 21.08	110.94 ± 18.80	< 0.0001
HbA1c (%)	4.94 ± 0.78	5.08 ± 0.75	5.17 ± 0.78	0.014
Triglyceride (mg/dL)	150.15 ± 77.29	156.50 ± 83.83	200.82 ± 130.48	< 0.0001
Total cholesterol (mg/dL)	190.30 ± 38.16	192.47 ± 35.97	205.51 ± 50.56	0.034
LDL-C (mg/dL)	116.34 ± 33.45	117.04 ± 32.26	123.52 ± 52.88	0.436
HDL-C (mg/dL)	45.04 ± 11.75	44.78 ± 10.31	45.12 ± 11.55	0.944
Systolic blood pressure (mmHg)	110.23 ± 10.49	110.73 ± 10.66	110.76 ± 10.64	< 0.0001
Diastolic blood pressure (mmHg)	70.37 ± 10.11	70.64 ± 10.20	70.65 ± 10.26	0.002
Abdominal obese (WC > 90 cm) (%)	32.9	41.8	57.4	< 0.0001
Men				
Age (yr)	43.04 ± 6.73	42.48 ± 6.20	43.85 ± 7.16	0.71
Energy intake (kcal/d)	2384.54 ± 558.37	2176.44 ± 648.34	2536.88 ± 651.42	0.343
Weight (kg)	77.98 ± 12.95	79.43 ± 13.44	81.38 ± 6.13	0.53
Height (cm)	170.78 ± 6.47	170.56 ± 6.30	169.55 ± 5.22	0.802
Body mass index (kg/m^2)	26.66 ± 3.53	27.22 ± 3.82	28.32 ± 1.82	0.201
Waist circumference (cm)	92.63 ± 9.43	93.14 ± 9.86	97.65 ± 7.64	0.19
Hip circumference (cm)	102.76 ± 6.68	103.91 ± 7.76	108.15 ± 7.19	0.025
Waist to hip ratio	0.90 ± 0.06	0.89 ± 0.05	0.90 ± 0.03	0.754
Fasting blood sugar	87.55 ± 8.25	90.34 ± 6.91	92.54 ± 4.11	0.005
Blood sugar after 30 min (mg/dL)	133.20 ± 28.96	140.95 ± 28.86	144.42 ± 31.05	0.091
Blood sugar after 60 min (mg/dL)	128.50 ± 35.76	136.26 ± 35.35	147.69 ± 44.80	0.081
Blood sugar after 120 min (mg/dL)	91.18 ± 24.14	92.40 ± 21.22	110.08 ± 20.20	0.019
HbA1c (%)	4.94 ± 0.66	5.09 ± 0.89	5.15 ± 1.01	0.293
Triglyceride (mg/dL)	178.85 ± 97.60	177.54 ± 92.37	219.23 ± 101.25	0.328
Total cholesterol (mg/dL)	189.49 ± 383.49	189.45 ± 31.95	204.31 ± 51.08	0.312
LDL-C (mg/dL)	114.74 ± 30.58	113.56 ± 31.07	118.60 ± 43.79	0.87
HDL-C (mg/dL)	40.92 ± 12.01	41.49 ± 9.21	45.92 ± 12.16	0.301
Systolic blood pressure (mmHg)	110.46 ± 10.51	110.87 ± 10.75	120.69 ± 10.48	0.011
Diastolic blood pressure (mmHg)	70.48 ± 10.13	70.83 ± 10.30	80.08 ± 10.08	0.04
Abdominal obese (WC > 90 cm) (%)	59.1	63.5	92.3	0.058
Women				
Age (yr)	41.70 ± 5.92	42.81 ± 6.21	42.94 ± 5.86	0.059
Energy intake (kcal/d)	1738.47 ± 488.91	1674.05 ± 491.68	1715.73 ± 447.60	0.643
Weight (kg)	70.05 ± 11.11	71.17 ± 11.30	77.06 ± 13.36	0.002
Height (cm)	156.52 ± 5.12	156.96 ± 5.25	156.27 ± 6.59	0.43
Body mass index (kg/m^2)	28.58 ± 4.26	29.23 ± 4.19	31.47 ± 4.45	< 0.0001
Waist circumference (cm)	85.10 ± 8.88	87.00 ± 9.16	91.03 ± 9.02	< 0.0001
Hip circumference (cm)	107.37 ± 8.79	108.68 ± 8.79	111.21 ± 9.94	0.02
Waist to hip ratio	0.79 ± 0.05	0.80 ± 0.05	0.82 ± 0.03	0.018
Fasting blood sugar	86.87 ± 7.91	88.89 ± 7.05	90.41 ± 6.71	0.001
Blood sugar after 30 min (mg/dL)	125.21 ± 23.32	134.55 ± 25.26	140.42 ± 23.59	< 0.0001
Blood sugar after 60 min (mg/dL)	120.96 ± 30.41	136.16 ± 29.46	153.38 ± 33.60	< 0.0001
Blood sugar after 120 min (mg/dL)	100.58 ± 19.45	109.63 ± 18.99	111.26 ± 18.55	< 0.0001
HbA1c (%)	4.94 ± 0.82	5.08 ± 0.68	5.18 ± 0.69	0.05
Triglyceride (mg/dL)	140.11 ± 66.06	148.32 ± 79.00	193.34 ± 141.41	< 0.0001
Total cholesterol (mg/dL)	190.74 ± 39.60	193.63 ± 37.41	206.00 ± 51.16	0.092
LDL-C (mg/dL)	117.04 ± 34.27	118.31 ± 32.67	125.64 ± 56.94	0.44
HDL-C (mg/dL)	46.48 ± 11.34	45.99 ± 10.45	44.77 ± 11.48	0.658
Systolic blood pressure (mmHg)	110.15 ± 10.48	110.67 ± 10.63	110.40 ± 10.58	< 0.0001
Diastolic blood pressure (mmHg)	70.33 ± 10.10	70.56 ± 10.15	70.48 ± 10.31	0.044
Abdominal obese (WC > 90 cm) (%)	24.1	33.3	44.1	0.004

[1]Values are mean ± SD. LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; HbA1c: Glycosylated haemoglobin.

Table 2 Crude and multivariable-adjusted relative risk and 95%CI for relative risk obtained from logistic regression

	Total		Men		Women	
	Pre-diabetes	Diabetes	Pre-diabetes	Diabetes	Pre-diabetes	Diabetes
BMI						
Crude	1.03 (1.00, 1.07)	1.14 (1.07, 1.21)	1.04 (0.97, 1.13)	1.14 (0.97, 1.34)	1.04 (1.00, 108)	1.15 (1.07, 1.23)
Model 1	1.04 (1.00, 1.07)	1.14 (1.06, 1.22)	1.14 (0.97, 1.35)	1.04 (0.97, 1.12)	1.03 (0.99, 1.07)	1.14 (1.06, 1.23)
Model 2	1.06 (0.98, 1.15)	1.24 (1.06, 1.46)	1.06 (0.80, 1.40)	1.30 (0.75, 2.26)	1.07 (0.98, 1.17)	1.25 (1.02, 1.53)
Model 3	1.05 (0.96, 1.14)	1.36 (1.05, 1.77)	1.00 (0.75, 1.35)	1.44 (0.42, 4.88)	1.07 (0.97, 1.17)	1.44 (1.05, 1.98)
Model 4	1.04 (0.96, 1.14)	1.38 (1.05, 1.82)	1.00 (0.75, 1.35)	Inestimable	1.07 (0.97, 1.18)	1.51 (1.06, 2.14)
WC						
Crude	1.02 (1.00, 1.03)	1.06 (1.03, 1.09)	1.01 (0.98, 1.03)	1.06 (1.00, 1.13)	1.02 (1.01, 1.04)	1.07 (1.03, 1.11)
Model 1	1.02 (1.00, 1.03)	1.07 (1.03, 1.10)	1.01 (0.98, 1.03)	1.06 (1.00, 1.13)	1.02 (1.00, 1.04)	1.07 (1.03, 1.11)
Model 2	1.04 (1.00, 1.08)	1.16 (1.06, 1.27)	1.00 (0.90, 1.12)	1.08 (0.87, 1.33)	1.05 (1.01, 1.09)	1.20 (1.07, 1.35)
Model 3	1.03 (0.99, 1.07)	1.20 (1.04, 1.38)	0.97 (0.85, 1.09)	1.58 (0.37, 6.76)	1.04 (1.00, 1.09)	1.21 (1.03, 1.42)
Model 4	1.03 (0.99, 1.07)	1.20 (1.04, 1.38)	0.97 (0.86, 1.09)	Inestimable	1.04 (1.00, 1.09)	1.22 (1.03, 1.45)

Model 1: Adjusted for age, and sex only in the whole population; Model 2: Further adjustment was made for physical activity and energy intake; Model 3: Further adjustment was made for blood sugar and HbA1c; Model 4: Further control was made for lipid profile and blood pressure; BMI: Body mass index; WC: Waist circumference.

Table 3 Area under the curve (95%CI for area under the curve) for body mass index and waist circumference on predicting the pre-diabetes or diabetes

	Total		Men		Women	
	Pre-diabetes	Diabetes	Pre-diabetes	Diabetes	Pre-diabetes	Diabetes
Body mass index	0.541 (0.502, 0.581)	0.673 (0.596, 0.749)	0.538 (0.460, 0.617)	0.664 (0.551, 0.778)	0.544 (0.498, 0.590)	0.691 (0.598, 0.784)
Waist circumference	0.552 (0.513, 0.592)	0.674 (0.602, 0.746)	0.508 (0.432, 0.585)	0.613 (0.505, 0.721)	0.564 (0.518, 0.611)	0.691 (0.604, 0.778)

among those who developed (affected by) diabetes.

There were 45 incident cases of physician-diagnosed diabetic patients during follow up from 2003 to 2013. Overall, there was a positive link between BMI and WC in crude and all adjusted models (Table 2). After controlling for various confounders and mediators, relative risk for diabetes increased by 38% for 1 s.d. increase in BMI (95%CI: 1.05-1.82, P = 0.019). One s.d. increase in WC was associated with 20% higher risk for developing diabetes (95%CI: 1.04-1.38, P = 0.010), after controlling for potential confounders and mediators. In men, 1 s.d. increase either in BMI or in WC could not significantly affect the risk of prediabetes and diabetes; however, in women, 1 s.d. increase in both BMI and WC were associated with higher risk of diabetes, but not prediabetes. In the full adjusted model, 1 s.d increase in BMI and WC increased risk of diabetes by 51% (95%CI: 1.06-2.14) and 22% (95%CI: 1.03-1.45) in women, respectively.

The AUCs (and 95%CIs) of BMI and WC in the prediction of pre-diabetes and diabetes are shown in Table 3. AUCs for both measures were larger for diabetes rather than pre-diabetes. As can be seen the significant predictive roles were detected for both BMI and WC on predicting diabetes while positive but not significant for prediabetes. Nevertheless, AUC of WC did not differ substantially from AUC of BMI either for pre-diabetes or for diabetes in the whole population. When analyses were run for men and women separately, similar results were obtained. Figure 1 supports the similar predictive powers of BMI and WC in predicting the incidence risk of diabetes in the whole population and either gender.

Table 4 indicates the optimal cutoff points for general obesity and abdominal adiposity to predict incidence of pre-diabetes and diabetes. Defined cut-off points based on maximum sensitivity plus specificity values suggested that in men, BMI of 26.2 kg/m^2 and WC of 89.7 cm, and in women, BMI of 28.6 kg/m^2 and WC of 84.3 cm would predict the incident risk of diabetes. In the whole population, BMI of 28.5 kg/m^2 and WC of 86.25 and 86.75 cm had the highest maximum sensitivity plus specificity. The optimal cutoff points for BMI to predict pre-diabetes in the whole population, men and women were 28.3, 29.6, 28.3 kg/m^2, respectively. Corresponding values for WC were 86.0, 89.7, 88.2 cm, respectively.

DISCUSSION

In this prospective study, BMI was strongly associated with diabetes incidence in the whole population and women. WC was moderately related to diabetes incident in the whole population and women. These associations remained significant after controlling either for confounding variables or mediators. The associations of BMI and WC with incidence of diabetes in men were not significant, and in overall both BMI and WC were weakly correlated with pre-diabetes incidence.

Adjustment for mediators increased the risk of

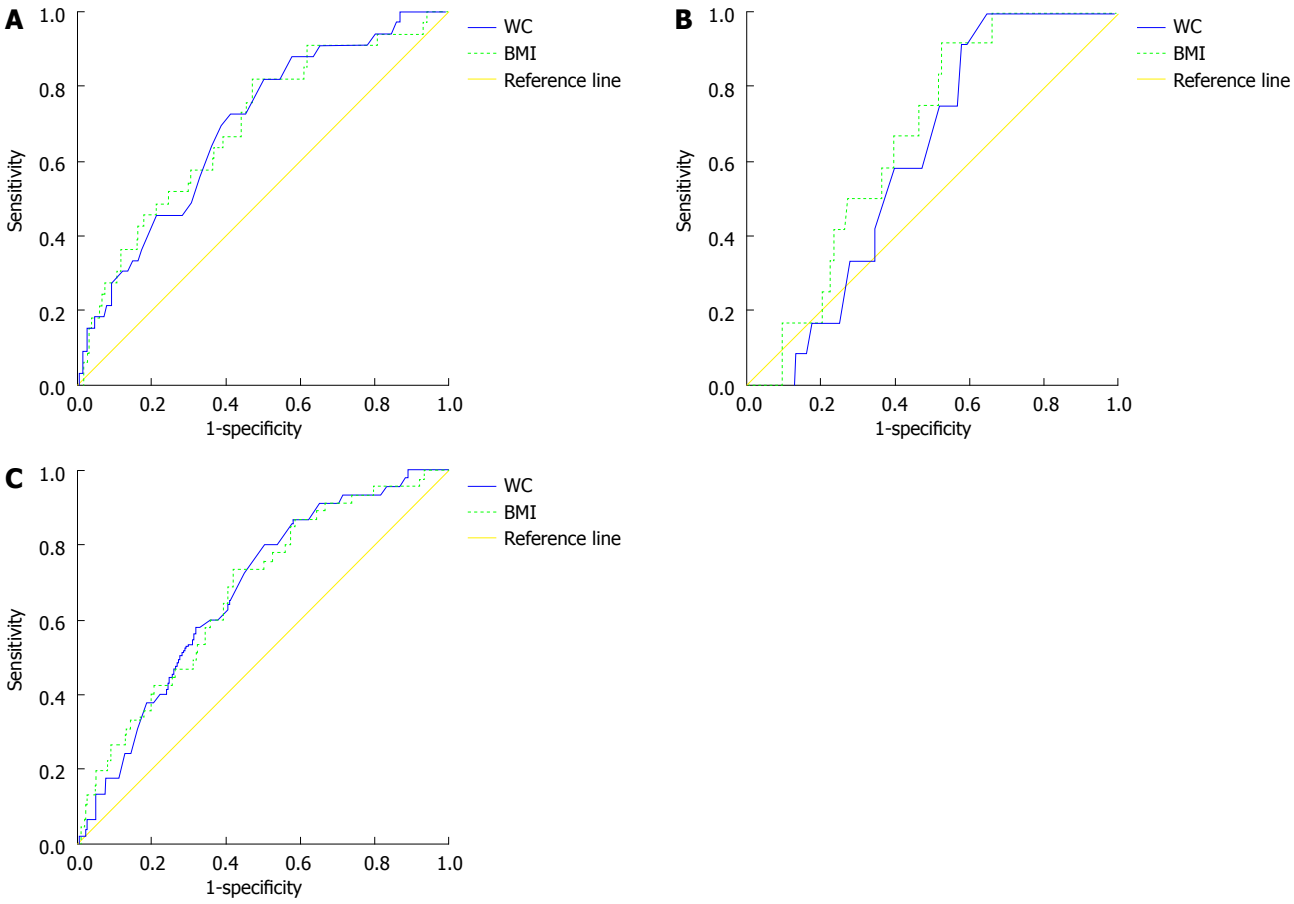

Figure 1 Comparison of receiver-operating characteristic curves for waist circumference (continuous line) and body mass index (dashed line) in women (A), men (B) and the whole population (C).

Table 4 Optimal cutoff points for general obesity and abdominal adiposity to predict incidence of pre-diabetes and diabetes

	Whole population		Men		Women	
	BMI	WC	BMI	WC	BMI	WC
Diabetes						
Cutoff point 1	28.5	86.25	26.2	89.7	28.6	84.3
Sensitivity	0.733	0.787	0.917	1	0.818	0.818
Specificity	0.579	0.495	0.477	0.356	0.53	0.5
Cutoff point 2	29	86.75	27	90	29	85
Sensitivity	0.6	0.766	0.75	0.917	0.667	0.758
Specificity	0.614	0.514	0.53	0.423	0.566	0.532
Pre-diabetes						
Cutoff point 1	28.3	86	29.6	89.7	28.3	88.2
Sensitivity	0.528	0.787	0.294	0.706	0.583	0.45
Specificity	0.559	0.495	0.819	0.356	0.516	0.686
Cutoff point 2	29	87	30	90	29	89
Sensitivity	0.427	0.723	0.235	0.635	0.468	0.376
Specificity	0.614	0.453	0.826	0.409	0.564	0.718

BMI: Body mass index; WC: Waist circumference.

diabetes incidence which might be attributed to adiposity alone or adipocytokines or other unmeasured risk factors such as dietary intake, lifestyle, inflammatory factors and family history[22,23]. Effects of these variables on anthropometric measures have been well-established[24,25]. In the DECODA (Diabetes Epidemiology: Collaborative Analysis of Diagnostic criteria in Asia) study, BMI and WC were not differently associated with the incidence risk of diabetes in men, but in women, WC was stronger anthropometric predictor of diabetes than BMI[7]. A meta-analysis of the Asian cohorts suggested that BMI and WC were similarly related to incident of diabetes[26]; however, in European, WC is stronger predictor for developing

diabetes than BMI[8]. Using prospective analyses, similar associations were found for BMI and WC to predict the progression of diabetes in an Iranian population[10]. Nevertheless, in the current analyses, we observed that BMI was a stronger predictor for the incident diabetes in women and the whole population. The discrepancies regarding BMI and WC relation with diabetes in the current analyses might reveal that the length of follow-up duration might be a relevant determinant of estimating incident risk. Regardless of the contradictory results on which of anthropometric measures is better, all studies indicated that both BMI and WC are directly associated with the incidence risk of diabetes. For pre-diabetes, in the whole population, cut points were similar to cut points of diabetes. In men, in spite of similar cut points for WC to predict pre-diabetes *vs* diabetes, there was considerable difference for BMI while greater BMI value was identified as the best cut point. In contrast, in women, BMI cut point for predicting pre-diabetes was similar to the one for diabetes, but WC cut point was considerably higher. This finding confirms that WC in women and BMI in men are better index for predicting pre-diabetes as well as for diabetes.

To determine appropriate cutoff points for anthropometric measures, some variables such as age and sex need to be taken into account. In men and older age adults, higher cut-off points are more suitable. However, in the current study, we determined cut-off points only based on sex since majority of participated subjects at baseline were younger than 60 years which is defined as the age of elderly[27].

In this study, there was no difference in the overall predictive discrimination (as determined by AUC) of BMI and WC in either gender, that is in line with other studies[28,29]. Yoon *et al*[29] indicated that BMI and WC have similar predictive power for insulin resistance and diabetes among Korean adults. Another population-based cross-sectional study on Iranian men and women aged 20-80 years found no difference in the predictive power of BMI and WC for diabetes[28]. Nevertheless, Johnston Alperet *et al*[30] revealed that central obesity measures (WC and waist to height ratio) are better than BMI for the diagnosis of uninvestigated diabetes mellitus in three major Asian ethnic groups (Chinese, Malays, and Asian-Indians).

Available evidences to determine suitable cut-off points for WC and BMI have been obtained from cross-sectional studies[12,13,16]. To the best of our knowledge, there is only one prospective cohort study to predict appropriate cut-off points for diabetes among Iranians[10]. Difference in study design may lead to inconsistency regarding the determined clinically relevant cut-off points in different studies. Moreover, the follow-up duration and the sample size of study may influence these cut points in studies with similar design. Our study suggested that in the whole population, the BMI cut points of 28.5 kg/m^2 and 86 cm for WC yielded the maximum sensitivity plus specificity for predicting the presence of diabetes. Corresponding values in men were 26.2 kg/m^2 and 89.7 cm, and in women were 28.6 kg/m^2 and 84.3 cm, respectively. Generally, women had higher values of BMI but lower values of WC cut points; and this means that in women, central obesity performed better than BMI to predict diabetes risk, whilst in men BMI perform better. In this analysis, ROC analysis was run to identify cut points. It should be taken into account that ROC method is dependent on the distribution of anthropometric measures in the study population. On the other hand, increasing mean values of anthropometrics by corresponding higher distributions would automatically increase derived cut points by the ROC analysis[31]. Therefore, higher cut points of WC in men and BMI in women could be explained by the higher mean values of WC and BMI in men and women, respectively. Moreover, ROC method is equally weighted for sensitivity and specificity[32]. This might lead to low sensitivity for anthropometric measures to predict the incidence risk of diabetes in clinical practice. Furthermore, defined cut points in our study could not be optimal points in clinic, since sensitivity *vs* specificity need to be weighed against other factors such as seriousness of the complaint, the applied test for evaluation (whether it is invasive or feasible) and how often the test must be done[33]. Furthermore, due to high prevalence of diabetes in Iran[25], it is relevant to identify a sensible proportion of the population at risk. Our defined cut-off points' sensitivity are higher than 80% that means only 20% diabetic subjects would be missed by these cut points. However, for pre-diabetes sensitivity is very low.

This study has several limitations that should be taken into account. The main limitation is few numbers of cases with diabetes that decrease the statistical power of analyses. Furthermore, our study population was not a representative sample of Iranians and therefore more studies are needed to confirm whether our findings are generalizable to other Iranian populations. In addition, a recent research has shown that non-alcoholic fatty liver disease (NAFLD) might be a new criterion for metabolic syndrome[34]. Regarding the high prevalence of NAFLD among Iranians[35] and due to its close relation with insulin resistance, further studies are needed to determine the suitable cut points for BMI and WC for predicting the NAFLD incidence among Iranians. Nevertheless, this study has some strength. Using measured anthropometric variables, not self-reported values, in a large sample of men and women with very reliable data are the main strengths of this study. Furthermore, confounding effects of various confounders and mediators were taken into account in data analyses. Finally, based on our prospective study design an association between fat accumulation and diabetes mellitus could be concluded.

In conclusion, we observed that the predictive powers of BMI and WC were similar in predicting the incidence risk of diabetes in either gender. The cut-off points for predicting diabetes in men and women were

different. Defined cut-off points based on maximum sensitivity plus specificity values suggested that in men, BMI of 26.2 kg/m^2 and WC of 89.7 cm, and in women, BMI of 28.6 kg/m^2 and WC of 84.3 cm would predict Isfahanian population at high risk for developing diabetes.

COMMENTS

Background

The increased prevalence of obesity in the world is a major concern as it is strongly related to multiple metabolic disorders, among them diabetes. Therefore, it is essential to identify the best anthropometric index and effective risk thresholds for adiposity measures to develop appropriate preventive strategies in each population.

Research frontiers

Current study aimed to prospectively determine the effective anthropometric measures to predict the risk of type 2 diabetes and prediabetes also estimate the optimal cut-off point of body mass index (BMI) and waist circumference (WC) by following up non-diabetic participants at baseline examination.

Innovations and breakthroughs

To the best of our knowledge, there is only one longitudinal study among Iranians which has determined cut-off point of WC for detecting cardiovascular disease risk and no study on BMI threshold, and other cut-off points come from cross-sectional surveys. on the other hand no study available on determining the best cut of values for prediabetes. These values are strengthening by the authors longitudinal study design.

Applications

Estimated cut-off points by the study will contribute to detect individuals at higher risk of developing type 2 diabetes as well as prediabetes.

Terminology

BMI and WC's best cut of values for predicting diabetes and prediabetes.

Peer-review

The article is an important epidemiological study in which the authors studied a cohort of 1765 healthy participants followed up from 2003 till 2013. The study is well conducted, with appropriate statistics methodology and the results, previously known, confirm the relationship between anthropometric data and diabetes mellitus.

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ORIGINAL ARTICLE

Prospective Study

Effect of bariatric surgery on adiposity and metabolic profiles: A prospective cohort study in Middle-Eastern patients

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Abstract

AIM

To investigate changes in adiposity and cardio-metabolic risk profile following Roux-en-Y gastric bypass in patients of Middle Eastern ethnicity with severe obesity.

METHODS

This prospective cohort study involved 92 patients who met the indications of bariatric surgery. Post-procedure markers of obesity and cardiometabolic profile were monitored regularly for a year.

RESULTS

Mean body mass index decreased by 29.5% from 41.9 to 29.5 kg/m^2 between baseline and 12-mo follow-up, while mean fat mass decreased by 45.9% from 64.2 kg to 34.7 kg. An improvement was also observed in the gluco-metabolic profile with both fasting glucose and HbA1c

substantially decreasing ($P < 0.001$).

CONCLUSION

The present study shows the short to medium term (1 year) health benefits of bariatric surgery for patients of Middle Eastern ethnicity.

Key words: Bariatric surgery; Anthropometric indices; Metabolic profile; Cardiometabolic risk

Core tip: The present study obviously shows the health benefits of Roux-en-Y gastric bypass bariatric surgery for the patients of Middle Eastern ethnicity, particularly during the first twelve months of follow-up.

Mazidi M, Rezaie P, Jangjoo A, Tavassoli A, Rajabi MT, Kengne AP, Nematy M. Effect of bariatric surgery on adiposity and metabolic profiles: A prospective cohort study in Middle-Eastern patients. *World J Diabetes* 2017; 8(7): 374-380 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i7/374.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i7.374

INTRODUCTION

Obesity and related complications pose increasing health challenges worldwide[1]. Obesity is associated with the development of various comorbidities including type 2 diabetes mellitus, hypertension, and dyslipidemia, which are well-documented risk factors for cardiovascular disease (CVD), as well as musculoskeletal disorders[2-5].

Dietary intervention, lifestyles modification and prescription of pharmaceuticals are the main methods of obesity prevention and control. However, nowadays there is growing attention to metabolic surgery/bariatric surgery, as a promising method to treat obesity. Roux-en-Y gastric bypass (RYGB) is a practical bariatric surgical procedure that has been shown to induce considerable weight loss in obese patients through restriction and malabsorption[6]. Improvement in insulin secretion and sensitivity; and consequently improvement in type 2 diabetes mellitus control after bariatric surgery have been reported in the clinical investigations[7,8]. Randomized controlled trials and observational studies support the medium-to-long term efficacy of RYGB in reducing body weight and fatness and controlling the major metabolic comorbidities of obesity[9,10]. But there still remain some uncertainties about the effects of bariatric surgery on cardiometabolic profiles of obese individuals especially in the Middle East countries and available studies on the efficacy of bariatric surgery mostly originate from the West and predominantly involve Caucasian or African American patients, while a growing number of people with severe obesity is increasingly found in developing countries[11]. Asian subjects have a different relationship between obesity and diabetes risk to Caucasians and hence the impacts of bariatric surgery in Asian populations may also differ from the effects reported previously in Caucasians. For example, Capella *et al*[12] reported when they looked at patient sub-populations, they found that Afro-Americans lost significantly less weight than Hispanic Americans or White Americans. In addition, Hispanic American women lost less weight than White American women.

To the best of our knowledge, there is no study to investigate the effect of bariatric surgery in Iranian and Middle Eastern ethnicity, hence the aim of the current study was to investigate the impact of the bariatric surgery on adiposity and metabolic profiles in the patients with Middle Eastern ethnicity.

MATERIALS AND METHODS

Study design, setting, and participant

This prospective observational cohort study was conducted between 2011 and 2015 at the Qaem and Imam Reza hospitals of Mashhad, Iran. A total of 92 participants (35 women, 38.0%) who had a body mass index (BMI) greater than 40 kg/m^2 (or more than 35.40 kg/m^2 with severe comorbidities due to obesity), aged between 25 and 65 years took part in the study. They all met the criteria for performance of bariatric surgery[13]. Pregnant or breast feeding women, patients with known malignancies and those with any condition precluding surgery or general anesthesia were excluded from the study. None of the patients had previously undergone bariatric surgery.

Baseline evaluations

Height (to the nearest 0.1 cm) was measured using a portable stadiometer (OTM, Tehran, Iran) in the upright position, without shoes, with the subject stretching to the maximum height and the head positioned in the Frankfurt plane. The weight and body composition were measured by a bio-impedance analyzer (BIA) (Tanita BC-418 MA, Tanita Corp., Japan) and participants were dressed in light clothing (*i.e.,* no shoes, sweaters or jackets, with 0.1 kg accuracy, frequency range 50-60 Hertz)[14]. The BIA was calibrated according to the manufacturer's guidelines before each testing and the participants were informed in advance not to use any substance affecting their body composition (*e.g.,* alcohol and coffee) 24 h before the test[14].

Body composition (weight, fat mass, free fat mass) was determined by bioelectric impedance using a Tanita Body Composition Analyzer (Tanita Corporation, Tokyo, Japan). Blood pressure was measured from the dominant arm, with subjects in a sitting position, after 10 min of rest. Measurements were repeated 3 times at 2-min intervals and the means of the 3 measurements were recorded.

For each participant, blood samples were drawn

into serum-separating tubes after an overnight fast. Part of the sample was used immediately to measure fasting plasma glucose by the glucose-oxidase, GOD-PAP method[15] and plasma lipids [cholesterol and triglyceride, high-density lipoprotein (HDL) and low-density lipoprotein (LDL)] were measured using enzymatic methods. Aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed using standardized methods [spectrophotometry corresponding commercial kits (Pars Azmoon, Iran)][16]. Total bilirubin and HbA1c were measured by commercially provided kits (Pars Azmun, Iran).

Index surgical procedure and follow-up evaluations

All 92 participants underwent a RYGB procedure following the same surgical technique performed by a single surgeon[17]. Patients were NPO (Nil per OS) for three days after the surgery; thereafter, liquid diet was carefully started after a swallow test with gastrografin, and was maintained for two weeks. Upon discharge, patients were followed at the outpatient clinics of Qaem and Imam Reza hospitals, Mashhad, each 3 mo for 12 mo with repetition of the assessments performed at baseline under the supervision of nutritionist and medical group.

Ethical consideration

All patients were completely informed about the surgical procedure offered including potential advantages, probable complications and cost-benefit ratio and completed a written informed consent to participate in the study. The study protocol was approved by the Mashhad University of Medical Sciences' Ethics Committee.

Statistical analysis

SPSS software (version 11.5, Chicago, IL, United States) was used for statistical analysis. Kolomogrov-Smirnov tests were used to evaluate the normality of data. Values are expressed as mean ± SD for normally distributed variables. For normally distributed variables paired *t*-test was used to compare the before and after surgery and equivalent test was used for the skewed variables. Change in adiposity markers and cardio-metabolic profiles during follow-up were investigated using the analysis of variance (ANOVA) and Kruskal-Wallis tests for repeated measures. *P*-value $\leqslant$ 0.05 was considered significant.

RESULTS

Baseline adiposity and change during the first 12 mo of follow-up

The mean BMI was 41.9 ± 4.5 kg/m^2 at baseline and steadily decreased down to 29.5 ± 3.8 kg/m^2 at 12 mo, giving a relative change of -29.5%. The relative change in BMI from baseline was -11.4% at 3 mo and -18.8% at 6 mo. The mean fat mass was 64.2 ± 11.0 kg at baseline and decreased by 45.9% down to 34.7 ± 8.2 kg at 12 mo. The relative change in fat mass was -21.0% at 3 mo and -37.5% at 6 mo (Table 1). In analyses stratified by gender, the patterns were very similar in men and women. For instance, the mean BMI decreased from 42.4 ± 5.2 kg/m^2 at baseline to 29.4 ± 4.5 kg/m^2 at twelve months in men, and from 41.1 ± 6.1 kg/m^2 to 29.6 ± 3.3kg/m^2 in women.

Baseline cardiometabolic risk profile and trajectories during the first 12 mo of follow-up

HDL increased ($P < 0.001$) while other cardiovascular risk factors including total cholesterol, LDL cholesterol, triglycerides and Hs-CRP levels steadily decreased ($P < 0.001$) between baseline and 12-mo follow-up, indicating an improvement of the cardiovascular risk profile (Table 1). An improvement was also observed in the gluco-metabolic profile with both fasting glucose and HbA1c substantially decreasing ($P < 0.001$) (Table 1). Both ALT and AST steadily decreased while total bilirubin increased during the first twelve months of follow-up ($P < 0.001$). Again, patterns were very similar in men and women taken separately.

DISCUSSION

For the first time in a population of Middle East ethnicity, this study evaluated the effect of bariatric surgery on adiposity indices and cardio-metabolic profiles in severely obesity subjects. We found that surgical intervention had a marked effect on adiposity which was substantiated by the significant decrease in BMI and fat mass during the first twelve months of follow-up. This was paralleled by significant and gradual improvement of the cardio-metabolic profiles over the same time period.

Consistent with our results, several studies have previously reported the beneficial effects of bariatric surgery on adiposity and cardio-metabolic profiles[18-20]. A prospective study conducted on 1156 severely obese participants in Utah reported that patients lost 27.7% of their initial body weight six years after RYGB surgery[21]. They also found that 94% of patients receiving RYGB surgery maintained at least 20% weight loss two years after surgery[21]. Observed weight loss in the Utah study was similar to the results of the Longitudinal Assessment of Bariatric Surgery (LABS) study[22]. Furthermore, in line with our findings, clinical studies with different age groups showed clinically meaningful weight loss and improvement in key health conditions among the participants who underwent bariatric surgery[23,24].

Considerable improvement of all lipid sub-fractions was observed during follow-up in our study, in line with other investigations[20,25,26]. The Swedish obese subject (SOS) study indicated that the incidence rate of hypertriglyceridemia was significantly lower in the surgically treated group than in the control group after

Table 1 Changes of anthropometrical and clinical factors from baseline to 12 mo follow up

Factors	Baseline (91)	3 mo (86)	6 mo (83)	12 mo (80)	*P* value
BMI (kg/m^2)	41.9 ± 4.5	37.1 ± 5.0	34.0 ± 4.6	29.5 ± 3.8	< 0.001
FM	64.2 ± 11.4	50.7 ± 9.2	40.1 ± 8.7	34.7 ± 8.2	< 0.001
FFM	68.3 ± 15.2	64.1 ± 15.8	60.7 ± 16.3	58.0 ± 16.6	< 0.001
LDL (mg/dL)	162.2 ± 9.7	147.9 ± 11.3	139.7 ± 13.2	122.8 ± 19.5	< 0.001
HDL (mg/dL)	35.7 ± 3.0	37.1 ± 3.4	36.9 ± 3.7	39.4 ± 5.3	< 0.001
TG (mg/dL)	232.6 ± 26.1	208.5 ± 31.3	168.2 ± 28.7	132.6 ± 25.8	< 0.001
TC (mg/dL)	244.1 ± 20.1	224.9 ± 24.8	198.2 ± 28.4	180.2 ± 42.7	< 0.001
FBG (mg/dL)	142.5 ± 18.1	115.1 ± 15.8	102.5 ± 10.1	97.1 ± 8.2	< 0.001
HbA1c (%)	6.8 ± 1.1	5.7 ± 0.92	5.5 ± 1.0	5.4 ± 1.2	< 0.001
ALT (IU/L)	44.8 ± 8.5	33.0 ± 9.6	30.1 ± 9.4	25.1 ± 8.3	< 0.001
AST (IU/L)	35.7 ± 7.0	28.1 ± 5.5	24.5 ± 5.2	23.5 ± 4.8	< 0.001
Total Bilirubin (mg/dL)	5.0 (3.2-6.1)	6.0 (4.1-8.5)	8.0 (5.5-9.5)	9.0 (6.1-11.8)	< 0.001
Hs-CRP (mg/dL)	24.0 (19.0-26.0)	20.0 (14.5-27.2)	18.1 (12.1-26.6)	7.7 (4.6-10.8)	< 0.001

Values expressed as mean ± SD for normally distributed data, and median and 25th-75th percentiles for non-normally distributed data. *P*-value is from the ANOVA or Kruskal-Wallis and it refers to total difference between follow up times. BMI: Body mass index; FBG: Fasting blood glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglyceride; TC: Total cholesterol; ALT: Alanine transaminase; AST: Aspartate transaminase; Hs-CRP: High-sensitivity C-reactive protein; FM: Fat mass; FFM: Fat free mass.

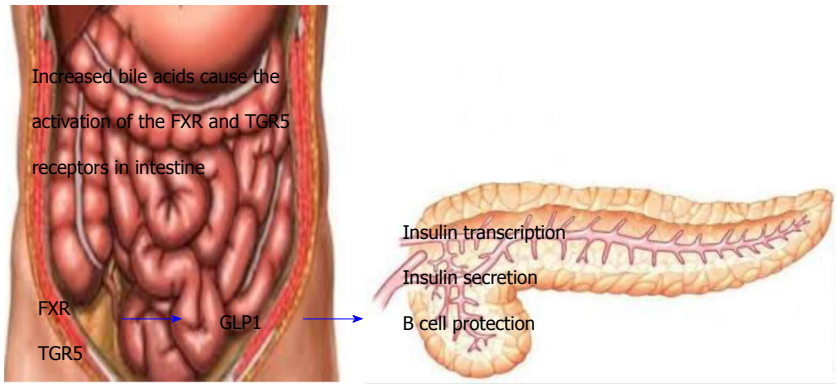

Figure 1 Increased concentration of bile acids caused to activation of farnesoid X receptor A and G protein-coupled bile acid receptor, these two receptors have adverse impact on different tissues. Activation of farnesoid X receptor A (FXRA) and G protein-coupled bile acid receptor (TGR5) can stimulate the secretion of glucagon-like peptide Ⅰ and Ⅱ (GLP), and GLP have positive effect on pancreas.

two years[27]. In addition, significant post-operative improvement of gluco-metabolic profiles was observed during follow-up in our study. Improvement of HbA_{1C} without medications has been reported in other studies[1,21,28].

In line with our findings, several pieces of evidence from clinical trials suggest that RYGB is associated with marked improvement in nonalcoholic fatty liver disease[29-31]. The long-term effect of bariatric surgery on liver enzymes in the Swedish Obese Subjects (SOS) study[32] indicated that bariatric surgery was related to lower serum ALT and AST levels at 2- and 10- year follow-up. In addition, analysis of the relation between changes in transaminase levels and changes in body weight indicated that weight gain was related to a substantial increase in transaminase levels.

In this study we have found that total bilirubin increased after the surgery, which is in line with other studies[33-42]. Very recently, Mazidi *et al*[43] have reviewed role of bile acids its subtractions on weight loss and glycaemic control after the bariatric surgeries. They have elaborated that there is a correlation between the concentration of the total bile acid and improvements in several key metabolic parameters after bariatric surgery[43]. It has been reported that there was an inverse correlation between bile acid concentrations and postprandial glucose and triglycerides, and a positive correlation with adiponectin and peak GLP-1 levels following a mixed meal test[33,44]. Moreover, augmented bile acid concentration (It has been suggested that changed upper intestinal tract structure after surgery might have an impact on the enterohepatic circulation of bile acids) could contribute to enhancements in insulin sensitivity, incretin secretion, lipid metabolism and postprandial glycemia after surgery[33,45]. As mentioned above, the release of GLP-1 is correlated with bile acids[43] (Figure 1). Therefore bile acid-dependent increases in postprandial GLP-1 concentrations may be somewhat responsible for the achievements of bariatric surgery in terms of both weight loss and glycaemic control[46].

Strengths and limitations

This study has strengths. The study is sufficiently powered to test the associations. We have repeated investigations of a range of adiposity and cardio-metabolic markers at baseline and during follow-up, which allowed us to carefully characterize their trajectories up to 12 mo after bariatric surgery. Moreover, it is one of the biggest studies which have

done in Middle East population. The findings from our study have to be considered in the context of some study limitations as well. We didn't collect data on the lifestyle of participants and are therefore unable to determine their contribution to some of the observed effects. Moreover, for evaluating the body composition dual-energy X-ray absorptiometry would be a better choice which we did not use it.

The present study suggests health benefits of RYGB bariatric surgery for the patients of Middle Eastern ethnicity, particularly during the first twelve months of follow-up. Our findings suggest that RYGB bariatric surgery has favorable short and medium term effects on adiposity and cardio-metabolic profiles in this population.

COMMENTS

Background

Roux-en-Y gastric bypass (RYGB) is a type of weight-loss surgery, bariatric surgery. It's often done as a laparoscopic surgery, with small incisions in the abdomen.

Research frontiers

This is the first and biggest study in middle-east subjects.

Innovations and breakthroughs

In this study they follow the subjects for 12 mo and through time, for each three months they have explore their adiposity and cardiometabolic factors.

Applications

Practical applications of the finding is that it can shed light on the post-operative side effcts and changes after the bariatrics surgery for such a novel population.

Peer-review

The article "Effect of bariatric surgery on adiposity and metabolic profiles. A prospective cohort study in middle-eastern patients", by Mohsen Nematy *et al* is a clinical prospective study on a cohort of middle eastern obese patients treated by RYGB. The alleged main difference between this and other similar studies is that the population was of Middle Eastern ethnicity.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Autologous bone marrow derived stem cell therapy in patients with type 2 diabetes mellitus - defining adequate administration methods

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Abstract

AIM

To carry out randomized trial for evaluating effects of autologous bone marrow derived stem cell therapy (ABMSCT) through different routes.

METHODS

Bone marrow aspirate was taken from the iliac crest of patients. Bone marrow mononuclear cells were separated

and purified using centrifugation. These cells were then infused in a total of 21 patients comprising three groups of 7 patients each. Cells were infused into the superior pancreaticoduodenal artery (Group Ⅰ), splenic artery (Group Ⅱ) and through the peripheral intravenous route (Group Ⅲ). Another group of 7 patients acted as controls and a sham procedure was carried out on them (Group Ⅳ). The cells were labelled with the PET tracer F18-FDG to see their homing and *in vivo* distribution. Data for clinical outcome was expressed as mean ± SE. All other data was expressed as mean ± SD. Baseline and post treatment data was compared at the end of six months, using paired *t*-test. Cases and controls data were analyzed using independent *t*-test. A probability (*P*) value of < 0.05 was regarded as statistically significant. Measures of clinical outcome were taken as the change or improvement in the following parameters: (1) C-peptide assay; (2) HOMA-IR and HOMA-B; (3) reduction in Insulin dose; subjects who showed reduction of insulin requirement of more than 50% from baseline requirement were regarded as responders; and (4) reduction in HbA1c.

RESULTS

All the patients, after being advised for healthy lifestyle changes, were evaluated at periodical intervals and at the end of 6 mo. The changes in body weight, body mass index, waist circumference and percentage of body fat in all groups were not significantly different at the end of this period. The results of intra-group comparison before and after ABMSCT at the end of six months duration was as follows: (1) the area under C-peptide response curve was increased at the end of 6 mo however the difference remained statistically non-significant (*P* values for fasting C-peptide were 0.973, 0.103, 0.263 and 0.287 respectively and the *P* values for stimulated C-peptide were 0.989, 0.395, 0.325 and 0.408 respectively for groups Ⅰ to Ⅳ); (2) the Insulin sensitivity indices of HOMA IR and HOMA B also did not show any significant differences (*P* values for HOMA IR were 0.368, 0.223, 0.918 and 0.895 respectively and P values for HOMA B were 0.183, 0.664, 0.206 and 0.618 respectively for groups Ⅰ to Ⅳ); (3) Group Ⅰ showed a significant reduction in Insulin dose requirement ($P < 0.01$). Group Ⅱ patients also achieved a significant reduction in Insulin dosages ($P = 0.01$). The Group Ⅰ and Group Ⅱ patients together constituted the targeted group wherein the feeding arteries to pancreas were used for infusing stem cells. Group Ⅲ, which was the intravenous group, showed a non-significant reduction in Insulin dose requirement ($P = 0.137$). Group Ⅳ patients which comprised the control arm also showed a significant reduction in Insulin dosages at the end of six months ($P < 0.05$); and (4) there was a non-significant change in the Hb A1c levels at the end of 6 mo across all groups ($P = 0.355$, $P = 0.351$, $P = 0.999$ and $P = 0.408$ respectively for groups Ⅰ to Ⅳ).

CONCLUSION

Targeted route showed a significant reduction in Insulin requirement at the end of six months of study period whereas the intravenous group failed to show reduction.

Key words: Autologous bone marrow derived stem cell therapy; Type 2 diabetes mellitus

Core tip: Homing of stem cells to pancreas is an important pre-requisite for achieving therapeutic efficacy in type 2 diabetes mellitus patients. Homing of stem cells was demonstrated when targeted infusion was carried out. No discernible homing was there when intravenous infusion route was employed. It was only in the targeted group that a 50% reduction in Insulin dosage was observed, establishing a relationship between homing and therapeutic efficacy.

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INTRODUCTION

Type 2 diabetes mellitus accounts for 90%-95% of all cases of diabetes mellitus and is caused due to insulin deficiency superimposed on insulin resistance[1,2]. The former is the major defect resulting in a blunted response of pancreatic beta cells to secrete Insulin in response to increased blood glucose levels. The resultant hyperglycaemic state is responsible for the microvascular and macrovascular complications seen in diabetic patients. Many patients require exogenous Insulin to control their blood glucose levels but even then it is difficult to achieve euglycemic state with both hyperglycemia and hypoglycaemia observed for variable times in a single day[3]. To circumvent this problem beta cell replacement therapy was sought as a viable alternative. It was hoped that this would provide a more physiological response of Insulin secretion to blood glucose levels. Islet cell transplantation was first tried on patients with type 1 diabetes mellitus. Despite the progress made in the ensuing time, this method suffered from inherent shortcomings like limited supply of pancreatic islets as 2-3 donors are needed for a single transplant, the need to give lifelong immuno-suppression and the progressive loss of implanted cells which necessitated the reintroduction of Insulin in these patients[4-7]. Also the utility of islet transplants in type 2 diabetics is not own. Because of the above reasons the adult stem cells taken from the patients' own bone marrow became the automatic choice for clinical trials and much of the current research has been based on utilizing the autologous cells for treating diabetics[8]. Various factors secreted by these cells

have been postulated to affect the angiogenesis and create a tissue micro environment that is conducive for beta cell regeneration and survival[9,10]. Though embryonic stem cells are more plastic as compared to adult stem cells, their use is limited because of the ethical concerns and safety issues.

The pertinent questions in regenerative medicine at present are to find out optimal routes for delivery of stem cells, optimal cell types and their numbers, and lastly to know how homing characteristics affect therapeutic efficacy. Aim of the present study was to carry out a clinical trial for evaluating the effects of autologous bone marrow derived stem cell therapy (ABMSCT) when they were infused through different routes and comparing them with a control arm. Analysis was carried out to find any association between the homing characteristics and therapeutic efficacy of infused stem cells.

Stem cells can be potentially transplanted utilising one of these three routes: (1) through targeted approach by injecting cells into the artery supplying the organ; (2) by putting cells into the peripheral vein; and (3) through direct injection into the organ. In the context of diabetic patients the first two approaches are commonly used for infusing stem cells[11]. There is risk of pancreatitis with direct injection into the pancreas and hence this approach has been used sparingly in clinical trials. In our study the labelled stem cells were tracked *in vivo* to see their biodistribution and to establish relationship between homing characteristics and therapeutic efficacy. *In vivo* tracking assumes importance because it is believed that the therapeutic efficacy of the infused cells will profoundly depend on delivery of these cells to the target organ, a process called homing[12] Subsequent maintenance of viability and functionality of these cells is also important for achieving the clinical goals.

It is imperative to know the vascular supply of pancreas for the targeted delivery of stem cells. The endocrine pancreas (body and tail of pancreas) in most parts is supplied by the branches of splenic artery[13]. The exocrine part of pancreas (pancreatic head) is supplied by the pancreatico-duodenal arteries[14].

Among the current imaging modalities PET is considered the preferred modality because of the advantages it offers; like convenience of labelling stem cells, minimal toxicity, accessibility, better sensitivity and resolution, signal being directly related to cell viability and its use in any model. Direct cell labelling with a radiotracer has been used for many years to track cells *in vivo*. Typically the cells in a solution are incubated with a radiotracer for a defined period during which the radiotracer gains access inside the cells. After the incubation the supernatant containing the unbound activity is removed. The labelled cells are injected into the host and tracked over time. Radio labelling with PET tracer F18-FDG is easily accomplished and does not require any cellular modification except for the need to fast the cells.

The cell survival and function is generally inferred by the measures of clinical outcome[15]. In case of diabetic patients these parameters are easily quantifiable and give objective evidence of therapeutic efficacy. Theoretically a targeted approach utilising the feeding arteries of the organ would ensure optimal delivery of these cells whereas the intravenous route infusion, though easily accomplished, is likely to fail in this endeavour[16-19].

MATERIALS AND METHODS

The subjects meeting the below mentioned inclusion and exclusion criteria were enrolled. Patients were randomized to different arms using computer generated randomisation table. All procedures were carried out according to the Institute's ethical guidelines. Statistical review of the study was performed by a biomedical statistician before the submission of draft. The statistical methods of this study were reviewed by Dr. Raman Chauhan from the department of Preventive and Social Medicine, IGMC, Shimla, Himachal Pradesh, India.

Inclusion Criteria: (1) Patients with T2DM between 30 and 70 years of age; (2) failure to triple OHA (Oral hypoglycaemic agents) and on stable doses of Insulin for at least 3 mo; (3) On Vildagliptin, Pioglitazone and Metformin for at least 3 mo along with Insulin to maintain euglycemia; (4) HbA1c of 6.5%-7.5%; (5) Insulin requirement $\geqslant$ 0.4 IU/kg per day; and (6) Glutamic acid decarboxylase (GAD 65) antibody negative status.

Exclusion Criteria: (1) Patients with T1DM or secondary diabetes; (2) Patients with serum creatinine > 1.5 mg/dL; (3) Abnormal liver function tests (defined as value of transaminases > 3 times the upper value of normal or serum bilirubin higher than normal for the reference value of the laboratory); (4) History of pancreatitis; (5) Seropositivity for HIV, HBsAg and HCV; (6) History of myocardial infarction or unstable angina in the previous 3 mo; (7) History of malignancy; (8) Patients with active infections; and (9) Female patients who are pregnant or lactating.

Design of the study: A total of 130 patients were screened from June 2010 to May 2012, out of which 42 were eligible for the study. They were randomly divided into four groups. A total of 28 cases were included in the final analysis. The cases were assigned to these four groups comprising 7 patients in each group. In Arm Ⅰ, 7 patients received stem cell infusion in the superior pancreatico-duodenal artery (Group Ⅰ), In Arm Ⅱ, 7 patients received stem cell infusion in the splenic artery (Group Ⅱ). In Arm Ⅲ, 7 patients were given stem cells through the peripheral intravenous route (Group Ⅲ). Another 7 patients acted as controls (Group Ⅳ).

Bone marrow aspirate was taken from the iliac crest of patients under aseptic precautions. Bone marrow mononuclear cells were separated and purified using centrifugation. The 2-3 mL of stem cell

concentrate obtained was anti-coagulated with heparin and then used for labelling with PET tracer F18-FDG.

The targeted injections were carried out under fluoroscopic guidance by the interventional radiologist employing digital subtraction angiography technique. In Group Ⅰ, a 5F catheter was selectively navigated through trans-femoral route into superior pancreatico-duodenal artery beyond the origin of cystic artery and super-selective injection of stem cells was carried out. Post stem cell transfer a diagnostic run was taken to look for the patency of superior pancreatico-duodenal artery. Similarly Group Ⅱ patients received super-selective injection in the proximal splenic artery at the origin of dorsal pancreatic artery. An F18-FDG PET scan was done at 30 min and 90 min after the infusion of F18-FDG labelled stem cells. One patient was imaged up to 16 h after the infusion; however there was no discernible tracer activity at the targeted region at that time. The Standardized uptake values (SUVs) in pancreas and other organs were determined for semi-quantitative analysis. Group - Ⅲ patients were imaged after peripheral intravenous injection of labelled stem cells was given in the ante-cubital vein.

Keeping the primary objectives in consideration at a power of 80% with confidence interval of 95%, the sample size for the study was calculated as 6 cases in single arm. The measures of clinical outcome were expressed as Mean ± SE. Baseline and post treatment data was compared at the end of six months, using paired *t* test. Cases and controls data were analyzed using independent *t* test. A probability (*P*) value of < 0.05 was regarded as statistically significant. Measures of clinical outcome were taken as the change or improvement in the following parameters: (1) C-peptide assay; (2) HOMA-IR and HOMA-B; (3) Reduction in Insulin dose - patients showing a reduction of more than 50% was regarded as responders; and (4) Reduction in HbA1c levels.

In all patients blood glucose was monitored intensively for 6 wk. Patients were asked to monitor at least two - 5 point profile blood sugar (defined as fasting, 2 h post breakfast, 2 h post lunch, 2 h post dinner and at 3 am) using glucometer every 2 wk. Dose escalation was done in first 2 visits. In the last 2 wk of run in period the patients were put on stable doses of Insulin which were taken as the baseline Insulin requirement. These patients received a fixed regime of three OHAs as follows: Vildagliptin 100 mg/d, Metformin 2 g/d and Pioglitazone 15 mg/d. The doses of OHAs were kept constant at this level and only Insulin dose was altered during the follow up period. Patients were evaluated at periodical intervals following stem cell therapy. Patients were urged to keep the five point profile of blood glucose levels and down titration of Insulin dosages was done on the basis of averaged out blood glucose values. HbA1c was measured at the end of 3 mo and at 6 mo. Maintenance or reduction of HbA1c values despite tapering of the insulin dosages was taken as significant. Insulin sensitivity indices of HOMA-IR and HOMA-B were calculated by the standard mathematical equations. After omitting Vildagliptin for 24 h and Insulin (NPH for 24 h and regular Insulin for 12 h) a baseline levels of: (1) fasting and Glucagon stimulated C-peptide and and (2) serum Insulin were done. HOMA-IR and HOMA-β was calculated using the following equations: - HOMA-IR C-peptide = fasting C-peptide × Fasting Plasma Glucose/22.5 - HOMA - β C-peptide = 20 × Fasting C-peptide/Fasting glucose - 3.5.

C-peptide is considered to be a good marker of Insulin secretion hence a marker of β - cell function because of its equi-molar secretion with Insulin, negligible hepatic extraction, and constant peripheral clearance at different plasma concentrations and in presence of alterations in plasma glucose concentrations. While fasting C-peptide alone is easy to obtain and correlates with stimulated C-peptide, it may be insufficient to detect subtle effects of therapy. After clinical diagnosis, the appropriate test may include the stimulated C-peptide response to intravenous glucagon. The stimulated C- peptide values were obtained at baseline and at the end of the study period. The area under C-peptide response curve was evaluated.

Comparison of baseline characteristics was carried out to check for fair randomisation and ensure that no bias creeps in at the time of reporting results (Table 1). Analysis of variance (ANOVA) was used to find p value. Procedural details in the three groups are shown in Table 2.

RESULTS

C-peptide assay and HOMA IR, HOMA B

The Insulin sensitivity indices among groups 1 to 4 are shown in Tables 3-6 respectively. The area under C-peptide response curve was increased at the end of 6 mo however the difference remained statistically non-significant across all groups (*P* values for fasting C-peptide were 0.973, 0.103, 0.263 and 0.161 respectively and the *P* values for stimulated C-peptide were 0.989, 0.395, 0.325 and 0.346 respectively).

The Insulin sensitivity indices of HOMA IR and HOMA B did not show any significant differences (*P* values for HOMA IR were 0.368, 0.223, 0.918 and 0.306 respectively). The *P* values for HOMA B were 0.183, 0.664, 0.206 and 0.242 respectively.

Insulin dose requirements

There was progressive and consistent decrease in fasting and post prandial plasma glucose leading to decrease in Insulin dosages after the targeted injection of stem cells into the feeding arteries of pancreas (superior pancreatico-duodenal and splenic arteries in Group 1 and Group 2 patients respectively). This reduction in Insulin dose remained statistically significant at 3 and 6 mo of follow up (*P* values of 0.011 and 0.003 respectively for Group 1 patients and

Table 1 Comparison of baseline characteristics

Parameter	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	*P* value
Age	57.83 ± 5.84	49.85 ± 9.63	53.28 ± 7.29	55.7 ± 7.7	0.351
Sex	M-4, F-3	M-6, F-1	M-6, F-1	M-5, F-2	0.592
Duration of diabetes (in years)	19.5 ± 5.54	14.28 ± 6.77	14.28 ± 5.64	19.6 ± 6.4	0.213
Insulin requirement	43.66 ± 5.35	39.71 ± 3.81	45 ± 6.57	43.86 ± 4.50	0.893
Duration of insulin (in years)	3.75 ± 1.72	4.47 ± 4.1	6.66 ± 4.54	4.4 ± 2.7	0.742
Weight	79 ± 18.89	72.14 ± 8.41	75.78 ± 9.95	77.7 ± 13	0.75
BMI	28.83 ± 4.26	26.57 ± 2.63	26.85 ± 3.97	29.6 ± 1.9	0.349
Body fat	31.5 ± 8.57	29.57 ± 5.38	33.14 ± 7.26	35.1 ± 2.5	0.452
Waist circumference	98.5 ± 9.46	92.57 ± 6.10	98.42 ± 9.51	101.6 ± 4.1	0.152
FPG	106.66 ± 14.36	103.42 ± 16.89	89.71 ± 9.21	103.5 ± 6.0	0.112
HbA1c	6.8 ± 0.18	6.4 ± 0.16	6.7 ± 0.15	6.6 ± 0.24	0.75
FPI (uU/mL)	123 ± 92	57 ± 48	22 ± 5	14.1 ± 4.3	0.17
HOMA-IR%	11.72 ± 5.39	3.31 ± 1.11	5.92 ± 0.67	4.6 ± 1.5	0.446
HOMA-B%	146.26 ± 56.44	40.75 ± 12.02	129.57 ± 65.20	77.8 ± 22.4	0.451
Fasting C-pep (ng/mL)	2.97 ± 1.51	1.28 ± 0.14	1.41 ± 0.25	1.1 ± 0.2	0.221
Stimulated C-peptide (ng/mL)	4.26 ± 1.59	2.63 ± 0.23	2.37 ± 0.36	2.1 ± 0.3	0.192
Number of BMMNCs infused	$4.9 \pm 3.10 \times 10^8$	$12.04 \pm 4.84 \times 10^8$	$6.88 \pm 2.30 \times 10^8$	NA	NA

BMI: Body mass index; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; HOMA-IR: Homeostasis model assessment insulin resistance index; BMMNCs: Bone marrow mononucleated cells; HOMA-B: Homeostasis model assessment beta cell function.

Table 2 Procedural details in Group Ⅰ, Ⅱ and Ⅲ patients were as follows

Parameter	Group Ⅰ	Group Ⅱ	Group Ⅲ
Volume of marrow aspirated (mL)	149 ± 60	222 ± 22.97	199 ± 49.91
Aspiration - Unilateral/Bilateral	U/L-3, B/L-4	B/L-7	U/L-4, B/L-3
Bone marrow TNC count	$11.72 \pm 6.36 \times 10^8$	$20.28 \pm 8.55 \times 10^8$	$11.72 \pm 3.03 \times 10^8$
Bone marrow MNC count	$4.9 \pm 3.10 \times 10^8$	$12.04 \pm 4.84 \times 10^8$	$6.88 \pm 2.30 \times 10^8$
Bone marrow CD 34+ cell count	0.71% ± 0.45%	0.87% ± 0.35%	0.58% ± 0.30%
Procedure time (min)	57 ± 18	50 ± 8	38 ± 18
Artery cannulated	SPD-5, SMA-2	Splenic-7	IV route
Catheter change required	1 case- multiple times	Nil	NA
SUV max at Pancreas at 30 min (mean ± SD)	21 ± 38	3.4 ± 3.34	1.56 ± 0.34

NA: Not applicable.

Table 3 Insulin sensitivity indices in Group Ⅰ

Time	0 mo (mean ± SE)	6 mo (mean ± SE)	12 mo	*P* value
FPI (uU/mL)	123 ± 91.87	75.83 ± 58.69		0.707
HOMA-IR%	11.72 ± 5.39	6.96 ± 1.32		0.368
HOMA-B%	146.26 ± 56.44	63.33 ± 13.99		0.183
Fasting C-peptide (ng/mL)	2.97 ± 1.51	2.94 ± 0.80		0.973
Stimulated C-peptide (ng/mL)	4.26 ± 1.59	4.23 ± 0.84		0.989

HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

Table 4 Insulin sensitivity indices in Group Ⅱ

Time	0 mo (mean ± SE)	6 mo (mean ± SE)	*P* value
FPI (uU/mL)	57.33 ± 48.19	71.04 ± 38.88	0.62
HOMA-IR %	3.31 ± 1.11	4.76 ± 0.45	0.223
HOMA-B %	40.75 ± 12.02	46.86 ± 2.62	0.664
Fasting C-peptide (ng/mL)	1.282 ± 0.14	1.896 ± 0.29	0.103
Stimulated C-peptide (ng/mL)	2.63 ± 0.23	3.008 ± 0.26	0.395

HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

P values of 0.003 and 0.01 respectively for Group2 patients). The intravenous arm patients belonging to

Table 5 Insulin sensitivity indices in Group Ⅲ

Time	0 mo (mean ± SE)	6 mo (mean ± SE)	*P* value
FPI (uU/mL)	21.94 ± 5.12	16.45 ± 4.39	0.434
HOMA-IR%	5.92 ± 0.67	6.13 ± 1.5	0.918
HOMA-B%	129.57 ± 65.20	59.58 ± 22.96	0.206
Fasting C-peptide (ng/mL)	1.415 ± 0.25	2.49 ± 0.74	0.263
Stimulated C-peptide (ng/mL)	2.37 ± 0.36	3.48 ± 0.87	0.325

HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

Table 6 Insulin sensitivity indices in Group Ⅳ

Time	0 mo (mean ± SE)	6 mo (mean ± SE)	*P* value
FPI (uU/mL)	14.1 ± 4.3	18.2 ± 4.3	0.525
HOMA-IR%	4.6 ± 1.5	4.9 ± 1.2	0.895
HOMA-B%	77.8 ± 22.4	91.7 ± 14.6	0.618
Fasting C-peptide (ng/mL)	1.1 ± 0.2	1.5 ± 0.4	0.287
Stimulated C-peptide (ng/mL)	2.1 ± 0.3	2.8 ± 0.8	0.408

HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

Table 7 Insulin dose (U/d) before and after autologous bone marrow derived stem cell therapy in Group I

Patient	Baseline	3 mo	6 mo	12 mo	18 mo	24 mo
1	32	22	16	Nil	Nil	Nil
2	66	55	53	53	50	_
3	30	20	16	14	-	-
4	46	26	20	20	-	-
5	40	18	12	-	-	-
6	48	8	10	-	-	-
7	Lost to FU					
Mean ± SE	44 ± 5	25 ± 7	21 ± 7			
P value		0.011	0.003			

Table 8 Insulin dose (U/d) before and after autologous bone marrow derived stem cell therapy in Group II

Patient	Baseline	3 mo	6 mo	12 mo
1	40	27	16	12
2	46	44	38	34
3	25	18	8	5
4	30	20	20	18
5	40	34	30	-
6	55	40	-	-
7	45	27	-	-
Mean ± SE	40 ± 4	30 ± 4	22 ± 5	
P value		0.003	0.01	

Group 3 did not show significant reduction at 3 mo and 6 mo of follow up (*P* value of 0.087 and 0.137 respectively). The Group 4 patients showed a non significant decrease at 3 mo (*P* value of 0.999) but a significant decrease at 6 mo of follow up (*P* value 0.021). This was ascribed to the placebo effect. When

Table 9 Insulin dose (U/d) before and after autologous bone marrow derived stem cell therapy in Group Ⅲ

Patient	Baseline	3 mo	6 mo	12 mo	18 mo
1	53	48	36	27	18
2	47	34	15	12	-
3	51	25	Nil	Nil	-
4	75	78	86	70	-
5	26	26	18	24	-
6	25	20	18	-	-
7	38	32	30	-	-
Mean ± SE	45 ± 7	38 ± 8	34 ± 11		
P value		0.087	0.137		

Table 10 Insulin dose (U/d) before and after autologous bone marrow derived stem cell therapy in Group Ⅳ

Patient	Baseline	3 mo	6 mo
1	30	30	32
2	37	25	23
3	46	40	34
4	68	55	57
5	41	26	20
6	45	41	39
7	40	49	24
Mean ± SE	44 ± 5	38 ± 4	33 ± 5
P value		0.999	0.021

cases and controls were compared there were no statistically significant differences in the Insulin dosage requirements. However the patients achieving a target of a 50% reduction in Insulin dosages and hence falling in the category of responders were seen only in the targeted group. Among different groups the insulin dose requirement before and after ABMSCT is depicted in Tables 7-10.

Inter and intra group comparison

When inter-group and intra-group comparisons were made for the parameters of clinical outcome across all groups, there were no statistically significant differences. The results are depicted in Tables 11 and 12.

HbA1c levels

The HbA1c levels were reduced in Group 1 patients despite tapering of the Insulin dosages (Mean value of 6.51 at six months compared to 6.65 at baseline). This group also showed best homing and retention of stem cells. Group 2 showed a slight increase in mean value of HbA1c, (6.7 at six months compared to 6.41 at baseline). However in this group also there was a significant decrease of Insulin requirement at the end of 6 mo. The Group 3 patients showed a non-significant decrease in the HbA1c levels (Mean value of 6.61 at six months compared to 6.7 at baseline). The group 4 patients maintained their HbA1c levels at 6.6 despite tapering of the Insulin dosages. The change in HbA1c levels among different groups is depicted in

Table 11 Comparison of different case arms (Group Ⅰ, Ⅱ and Ⅲ) with control arm (Group Ⅳ) at zero month

Parameter	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	*P* value Group Ⅰ	*P* value Group Ⅱ	*P* value Group Ⅲ
Insulin requirement	44 ± 5	40 ± 4	45 ± 7	44 ± 5	0.979	0.541	0.927
HbA1c	6.8 ± 0.18	6.4 ± 0.16	6.7 ± 0.15	6.6 ± 0.24	0.983	0.453	0.9
HOMA IR%	11.72 ± 5.39	3.31 ± 1.11	5.92 ± 0.67	4.6 ± 1.5	0.544	0.327	0.333
HOMA-B%	146.2 ± 56.44	40.75 ± 12.02	129.57 ± 65.20	77.8 ± 22.4	0.593	0.212	0.559
Fasting C-peptide (ng/mL)	2.97 ± 1.51	1.282 ± 0.14	1.415 ± 0.25	1.1 ± 0.20	0.174	0.087	0.139
Stimulated C-peptide (ng/mL)	4.26 ± 1.59	2.63 ± 0.23	2.37 ± 0.36	2.1 ± 0.3	0.144	0.132	0.324

HbA1c: Glycosylated hemoglobin; HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

Table 12 Comparison of different case arms (Group Ⅰ, Ⅱ and Ⅲ) with control arm (Group Ⅳ) at 6 mo

Parameter	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	*P* value Group Ⅰ	*P* value Group Ⅱ	*P* value Group Ⅲ
Insulin requirement	21 ± 7	22 ± 5	34 ± 11	33 ± 5	0.174	0.183	0.69
HbA1c	6.5 ± 0.16	6.7 ± 0.12	6.7 ± 0.21	6.6 ± 0.22	0.816	0.704	0.779
HOMA IR%	6.96 ± 1.32	4.76 ± 0.45	6.13 ± 1.5	4.9 ± 1.2	0.404	0.358	0.322
HOMA B%	63.33 ± 13.99	46.86 ± 2.62	59.58 ± 22.96	91.7 ± 14.6	0.096	0.29	0.213
Fasting C-peptide (ng/mL)	2.94 ± 0.80	1.896 ± 0.29	2.49 ± 0.74	1.5 ± 0.4	0.091	0.34	0.187
Stimulated C-peptide (ng/mL)	4.23 ± 0.84	3.008 ± 0.26	3.48 ± 0.87	2.8 ± 0.	0.14	0.566	0.412

HbA1c: Glycosylated hemoglobin; HOMA-IR: Homeostasis model assessment insulin resistance index; HOMA-B: Homeostasis model assessment beta cell function.

Table 13 Change in HbA1c % before and after autologous bone marrow derived stem cell therapy in Group Ⅰ

Patient	Baseline	3 mo	6 mo	12 mo	18 mo	24 mo
1	7.2	5.7	7.1	6.4	6.8	7.3
2	6.7	6.2	6.7	6.9	6	-
3	6	-	6.4	5.7	-	-
4	7	6.8	6.6	-	-	-
5	6.1	6.3	5.9	-	-	-
6	6.9	6.5	6.4	-	-	-
7	Lost to FU					
Mean ± SE	6.8 ± 0.18	6.3 ± 0.18	6.5 ± 0.16			
P value		0.164	0.355			

Table 14 Change in HbA1c % before and after autologous bone marrow derived stem cell therapy in Group Ⅱ

Patient	Baseline	3 mo	6 mo	12 mo	18 mo
1	7	7.1	7	7.4	6.5
2	6.2	6.4	6.5	6.7	7.7
3	6.6	6.5	6.5	6.5	-
4	5.6	7.5	7	6.4	-
5	6.6	6.7	6.5	-	-
6	6.4	6.3	-	-	-
7	6.5	5.7	-	-	-
Mean ± SE	6.4 ± 0.16	6.6 ± 0.21	6.7 ± 0.12		
P value		0.573	0.351		

Table 15 Change in HbA1c % before and after autologous bone marrow derived stem cell therapy in Group Ⅲ

Patient	Baseline	3 mo	6 mo	12 mo	18 mo
1	6.3	6.2	5.9	7.1	5.9
2	6.8	5.8	6.8	6.3	-
3	6.9	6.9	6.9	7.4	-
4	6.8	6.7	7.2	7	-
5	6.9	7.2	7.5	7.5	-
6	6	6	6.2	-	-
7	7.2	6.8	6.4	-	-
Mean ± SE	6.7 ± 0.15	6.5 ± 0.19	6.7 ± 0.21		
P value		0.28	0.999		

Table 16 Change in HbA1c % before and after autologous bone marrow derived stem cell therapy in Group Ⅳ

Patient	Baseline	3 mo	6 mo
1	6.6	6.3	5.5
2	7	7.1	6
3	7	7	6.7
4	7.5	7.8	7
5	6	6.9	7.1
6	6.8	7.2	6.8
7	5.6	5.5	7
Mean ± SE	6.6 ± 0.6	6.8 ± 0.7	6.6 ± 0.2
P value		0.258	0.408

Tables 13-16.

DISCUSSION

There has been a growing interest among the medical scientific community for utilizing cellular therapies in the treatment of Type 2 diabetes and its complications. An exponential rise in recent publications and clinical trials bears testimony to this fact. However despite the rapid transition from animal studies to clinical trials, many questions still remain unanswered in the field of regenerative medicine. Important ones being to find

the optimal cell delivery routes and techniques, to find optimal number and type of cells needed to achieve desired treatment effectiveness and also to find out the relationship between homing characteristics and therapeutic efficacy.

This is the first single blinded randomised case controlled study which evaluates the role of autologous bone marrow derived stem cells in patients with type 2 diabetes mellitus utilising different routes of administration and comparing them with a control arm. The study revealed that there was an increase in area under C-peptide response curve among all groups but the values remained statistically non-significant. No significant improvement in Insulin sensitivity indices of HOMA IR and HOMA B was noted. A progressive and consistent decrease in fasting and post prandial plasma glucose leading to decrease in Insulin doses was noted in group Ⅰ and group Ⅱ patients - the patients receiving targeted infusion of stem cells into the feeding arteries of pancreas. This reduction in Insulin dosages remained statistically significant at 6 mo of follow-up. The patients receiving intravenous infusion and belonging to group Ⅲ did not show any statistically significant reduction in Insulin requirements at the end of study period. The controls belonging to Group Ⅳ did have reduction in Insulin dose of 10 units (25%) at 6 mo. However none of them reached the primary objective of 50% reduction in Insulin dose. Though the reduction was statistically significant with a *P* value of 0.021, intensive lifestyle modifications after the sham procedure may have contributed to this reduction. HbA1c levels showed reduction or were maintained at the baseline level and this was observed despite tapering of Insulin dosages across all groups. However since the tapering of Insulin dosages were different across all groups and were not uniform, correlation coefficient for this parameter could not be calculated.

Maximum localization of labeled stem cells was seen in Group Ⅰ followed by Group Ⅱ patients. These groups included the patients which achieved the objective of 50% reduction in Insulin dose requirement at the end of study period. In the Group Ⅲ patients no discernible homing was there which corresponded to no significant change or improvement in the measures of clinical outcome. Hence it can be extrapolated that homing is tightly linked to therapeutic efficacy.

More studies are needed on this topic to validate the cost-effectiveness, durability, long term safety, molecular mechanisms involved and efficacy in varied population of type 2 diabetes patients.

Targeted route consistently showed an increase in area under C-peptide response curve. However no significant improvement in Insulin sensitivity indices of HOMA IR and HOMA B was noted. When the patients of this group were followed up for a period of six months, a significant reduction in the insulin requirement was noted. The HbA1c levels either showed reduction or were maintained at the baseline level despite tapering of insulin dosages.

On the other hand the intravenous group did not show any significant change or improvement in the parameters of clinical outcome. It was concluded that this route is not effective in type 2 diabetics.

Patients belonging to the control group showed a decrease in Insulin dose at 6 mo of follow up and this was ascribed to the placebo effect. However the probability of being a true responder was observed in the targeted group of patients only. Homing and retention of cells was documented when the targeted approach was used and was not seen when the cells were infused intravenously, lending credence to the theory that delivery of these cells to pancreas is an important prerequisite for achieving therapeutic efficacy.

COMMENTS

Background

Adult bone marrow mononuclear cells have shown promising therapeutic potential in various degenerative disorders including type 2 diabetes mellitus. However the optimal routes for infusing stem cells and their *in vivo* distribution have not been defined. The present study was designed to evaluate the effects of autologous bone marrow mononuclear cell therapy, when these cells were infused through different routes and comparing them with a control arm. Analysis was carried out to find any association between the homing characteristics and therapeutic efficacy of the infused cells.

Research frontiers

It is hard to imagine a more engaging contemporary research issue than stem cell research. Researchers believe that stem cell therapy has the potential to dramatically change the treatment of human disease. A number of adult stem cell therapies already exist, particularly bone marrow transplants that are used to treat leukaemia. The role of stem cell therapy is being probed in a host of other ailments and degenerative disorders including type 2 diabetes mellitus. The pertinent questions in regenerative medicine at present are to find optimal cell types, their dosages and the effective routes of administration.

Innovations and breakthroughs

A key issue to consider is the route of administration. A reliable stem cell delivery system is essential to the success of stem cell therapy. Most currently offered therapies are utilizing the intravenous delivery route for adult stem cells infusion. However there is a concern that stem cells might fail to reach to their targeted destination because they might get trapped in the lung. This is the first study of its kind which has sought to find out treatment outcome of autologous bone marrow mononuclear cell therapy by targeted approach vis-a-vis peripheral intravenous route and comparing them with a control arm.

Applications

The study demonstrated that the probability of being a responder was there when cells were delivered through intra-arterial route. Homing and retention of cells was seen when the targeted approach was used and was not seen in the intravenous approach. Hence it was concluded that delivery of these cells to pancreas is an important prerequisite for achieving therapeutic efficacy.

Terminology

Bone marrow derived stem cells: Bone marrow derived stem cells are multi-potent stem cells that are capable of trans-differentiating into cell types other than cells of hematopoetic lineage. These cells can migrate towards the site of damage and differentiate under the influence of factors from the local micro environment.

Peer-review

In the opinion very good piece of work - bone marrow derived stem cell therapy

well done Randomized Controlled Trial performed well.

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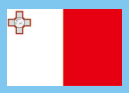

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Contents

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ORIGINAL ARTICLE

Retrospective Cohort Study

Observational Study

Randomized Clinical Trial

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prevalence of obesity and diabetes in patients with schizophrenia

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Abstract

AIM

To compare the prevalence of diabetes in patients with schizophrenia treated at a community mental health center with controls in the same metropolitan area and to examine the effect of antipsychotic exposure on diabetes prevalence in schizophrenia patients.

METHODS

The study was a comprehensive chart review of psychiatric notes of patients with schizophrenia and schizoaffective disorder treated at a psychosis program in a community mental health center. Data collected included psychiatric diagnoses, diabetes mellitus diagnosis, medications, allergies, primary care status, height, weight, body mass index (BMI), substance use and mental status exam. Local population data was downloaded from the Centers for Disease Control Behavioral Risk Factor Surveillance System. Statistical methods used were χ^2 test, Student's *t* test, general linear model procedure and binary logistic regression analysis.

RESULTS

The study sample included 326 patients with schizophrenia and 1899 subjects in the population control group. Demographic data showed control group was on average 7.6 years older (P = 0.000), more Caucasians (78.7% *vs* 38.3%, P = 0.000), and lower percentage of males (40.7% *vs* 58.3%, P = 0.000). Patients with schizophrenia had a higher average BMI than the subjects in the population control (32.11, SD = 7.72 *vs* 27.62, SD = 5.93, P = 0.000).

Patients with schizophrenia had a significantly higher percentage of obesity (58.5% *vs* 27%, *P* = 0.000) than the population group. The patients with schizophrenia also had a much higher rate of diabetes compared to population control (23.9% *vs* 12.2%, *P* = 0.000). After controlling for age sex, and race, having schizophrenia was still associated with increased risk for both obesity (OR = 3.25, *P* = 0.000) and diabetes (OR = 2.42, *P* = 0.000). The increased risk for diabetes remained even after controlling for obesity (OR = 1.82, *P* = 0.001). There was no difference in the distribution of antipsychotic dosage, second generation antipsychotic use or multiple antipsychotic use within different BMI categories or with diabetes status in the schizophrenia group.

CONCLUSION

This study demonstrates the high prevalence of obesity and diabetes in schizophrenia patients and indicates that antipsychotics may not be the only contributor to this risk.

Key words: Schizophrenia; Antipsychotic; Diabetes; Body mass index; Obesity

Core tip: This study compares obesity and diabetes rates between schizophrenia patients treated in a community mental health center and a local population control. It demonstrates that prevalence of obesity and diabetes is significantly higher in patients with schizophrenia, which is consistent with previous research. In this cross-sectional study, second generation antipsychotic use and antipsychotic dosage were not correlated with obesity categories or diabetes status. This implies that antipsychotics alone may not be responsible for the increased diabetes risk in schizophrenia patients. Many factors may contribute to risk, including an inherent vulnerability to diabetes in schizophrenia patients that has been seen in earlier studies.

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INTRODUCTION

It is now well established that people with serious mental illness (SMI), including schizophrenia, have excess morbidity and mortality leading to a reduced lifespan of 20-25 years compared with the rest of the population[1,2]. The increased mortality is largely attributable to physical illness, including metabolic abnormalities and cardiovascular disease, rather than factors that are directly associated with psychiatric illness such as suicide or homicide.

Schizophrenia is seen in approximately 1% of the population. The rates of obesity and diabetes in patients with schizophrenia are higher than the general population[3]. Obesity is reported in approximately 50% patients, metabolic syndrome is reported in up to 40%, glucose intolerance in up to 25% and diabetes in up to 15% patients with schizophrenia[4]. The cause for the increased prevalence of these conditions is multifactorial. Antipsychotics, a cornerstone of treatment in people with schizophrenia, cause weight gain, glucose intolerance and other metabolic complications. Second generation antipsychotics, notably clozapine and olanzapine, are associated with a 5-fold increase in metabolic syndrome after three years of treatment[5]. Patients with schizophrenia are known to have unhealthy diets and inadequate physical activity[6] due to lower socioeconomic status, lower educational level, and sub-optimal living situations. Symptoms of schizophrenia such as low motivation, apathy and cognitive deficits also could play a role in preventing access to high quality health care.

An additional important contributing factor to the increased prevalence of diabetes in schizophrenia may be an inherent susceptibility to diabetes in people with schizophrenia. Patients with schizophrenia have an increased risk of diabetes in family members[7,8]. Also, parental diabetes is a significant predictor of diabetes in people with psychotic disorders[8]. Inflammatory markers are seen in both schizophrenia and metabolic syndrome and the increased inflammation may explain the association between these conditions[9].

A recent meta-analysis of metabolic parameters in first episode psychosis patients demonstrated increased insulin resistance and impaired glucose tolerance when compared to controls[10]. However, an earlier review of diabetes in first episode patients showed that established diabetes was much less common in first-episode psychosis patients compared to those already on antipsychotics[11]. People with schizophrenia may have increased vulnerability manifesting as pre-diabetes that is compounded by cumulative exposure to antipsychotic medications.

The purpose of this study is to compare the prevalence of obesity and diabetes in patients with schizophrenia treated at a community mental health center with population controls in the same metropolitan area. The authors hypothesized that the prevalence would be higher in patients with schizophrenia. The study also examines the effect of antipsychotic exposure on diabetes prevalence in schizophrenia patients.

MATERIALS AND METHODS

Comprehensive psychiatric review notes of consecutive patients followed at Connecticut Mental Health Center (CMHC) Psychosis Program within a one-year period were audited. The study was approved by the Yale Human Investigations Committee. Inclusion criterion was a chart diagnosis of schizophrenia or schizoaffective disorder verified by the Structured Clinical Interview for

Table 1 Demographic characteristics

	Schizophrenia	Population control
n	326	1899
Age[a]	47.47 (± 26)	55.13 (± 18.21)
Sex (% male)[b]	58.30%	40.70%
Race[c]		
White	38.30%	78.70%
Black	49.70%	9.50%
Hispanic	6.40%	7.90%
Other	1.80%	3.90%
Diagnosis		
Schizophrenia	7%	
Schizoaffective	23.30%	
Antipsychotic medication (*n* = 306)		
First generation	81 (26.5%)	
Second generation	194 (63.4%)	
Both	31 (10.1%)	
Multiple antipsychotic use	59 (2.7%)	
Chlorpromazine equivalent dose	667.0 (± 507.9)	

[a]$P = 0.000$; [b]$P = 0.000$; [c]$P = 0.000$.

Diagnostic and Statistical Manual-IV (SCID for DSM-IV). CMHC Psychosis Program is a diagnosis specific, multidisciplinary outpatient clinic and research program, which serves community dwelling, low-income adult patients diagnosed with a non-affective psychosis. The program is the major point of care for psychotic patients who dwell in the Greater New Haven area, an urban catchment area with an estimated census of 200000 people. The program itself has a census ranging 450 to 500 patients, with about 5% annual turnover rate.

Comprehensive psychiatric review is a 30-50 min full psychiatric examination of established patients by one of four physicians and one advanced practice nurse. Notes are recorded in an institution specific standard form, and requires recording of all diagnoses, all medications, allergies, primary care and physical/gynecological exam status, review of systems, height, weight, body mass index (BMI), substance use, and mental status information. Per Psychosis Program policies, every patient is weighed before the exam with the same scale calibrated regularly and height is measured at least once with the same scale during patients' tenure in the clinic. While waist circumference is a better indicator of abdominal obesity and subsequent cardiovascular risk, it is not clear that it offers additional information for clinical management. Also it is not part of usual clinical care due to provider discomfort[12]. Hence, BMI was used as an index for obesity in this study. Diabetes mellitus (DM) type 2 diagnosis was extracted from the chart by self-report and verified by obtaining primary care records. In addition, patients were screened at least yearly for diabetes by glycosylated hemoglobin (Hba1c) and referred for treatment if they tested positive.

The control group is local population in Greater New Haven metro area and data are downloaded from United States Centers for Disease Control and Prevention (CDC) Behavioral Risk Factors Surveillance System (BRFSS)[13]. BRFSS is an ongoing telephone health survey tracking health conditions and risk behaviors in the United States. Details are described at www.cdc.gov/brfss/. Latest local data was from 2012.

De-identified patient and population data was merged in an SPSS v.22 data file, and corresponding variables recoded for compatibility. Categorical data was compared with χ^2 test, continuous data with Student's *t* test. General linear model (GLM) procedure was utilized to explore the relationship between BMI and schizophrenia while controlling for demographic factors. Binary logistic regression analysis was utilized to calculate adjusted odds ratios.

RESULTS

The study sample included 326 patients with schizophrenia and 1899 subjects in control group of local population. Demographic data for the population control and schizophrenia as well as clinical data for the schizophrenia subjects is presented in Table 1. Control group was on average 7.6 years older ($t = 7.36$, $P = 0.000$), included more subjects that identified themselves as Caucasian (78.7% *vs* 38.3%, $\chi^2 = 228.35$, $P = 0.000$), and had lower percentage of males (40.7% *vs* 58.3%, $\chi^2 = 35.02$, $P = 0.000$).

Subjects with schizophrenia had a higher average BMI (32.11, SD = 7.72) than the subjects in the control group (27.62, SD = 5.93). The difference was highly significant (4.49, 95%CI: 3.75, 5.23; $T = 11.83$, $P = 0.000$). When BMI categories were examined, schizophrenia group had a significantly higher percentage of obesity than the control group (58.5% *vs* 27%), and the control group had a higher percentage of normal and overweight subjects (Figure 1, $\chi^2 = 125.14$, $P = 0.000$). When obesity categories were examined among the obese subjects, schizophrenia group had a higher percentage of Class 2 and 3 obesity (*i.e.*, BMI between 35 to 40 and BMI > 40 respectively) than the control group (Figure 2, $\chi^2 = 6.13$, $P < 0.05$). Within the schizophrenia group, antipsychotic medication dosage was not significantly correlated with BMI either in the entire group or the obese group ($P = 0.93$ and 0.92 respectively). Also, there was no difference in the distribution of second generation antipsychotic use or multiple antipsychotic use within different BMI categories in the schizophrenia group ($P = 20.24$ and 0.19 respectively). Based on these findings, medication variables were dropped out of multivariable tests.

Given the differences between two groups in demographics, a univariate analysis was conducted and GLM procedure used to control for age, sex, and race. After controlling for these demographic variables, schizophrenia group still had a highly significant association with higher BMI ($F = 26.78$, df = 1, $P = 0.003$). None of the other variables, or interactions with demographic variables, were significant. Following this, a binary logistic regression analysis was conducted

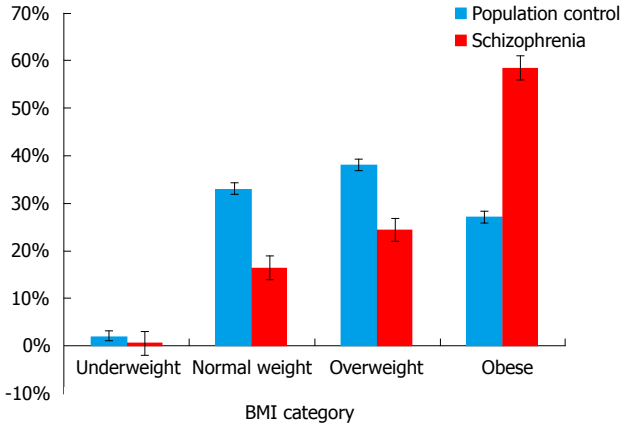

Figure 1 Body mass index categories for schizophrenia patients *vs* population (%). BMI: Body mass index.

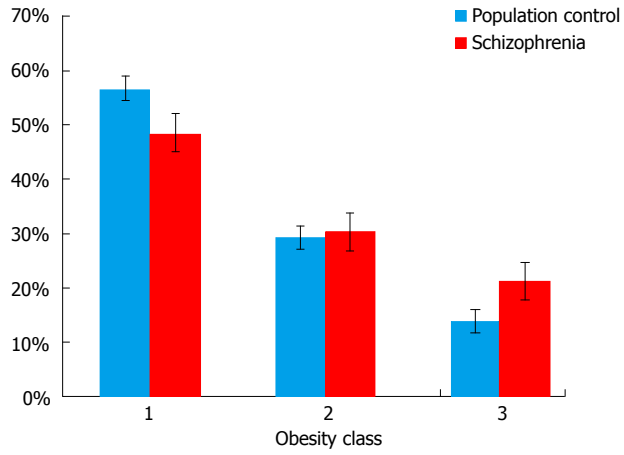

Figure 2 Obesity class categories; schizophrenia patients *vs* population (%).

to calculate predictive value of schizophrenia status for obesity after age, sex and race controlled, and schizophrenia status remained significant with an odds ratio of 3.25 (95%CI: 2.47, 4.29, P = 0.000).

Next, diabetes mellitus status was examined in the sample. Schizophrenia group had a much higher rate of diabetes compared to control group (Figure 3, 23.9% *vs* 12.2%, χ^2 = 31.81, P = 0.000). Within the schizophrenia group, there were no statistically significant differences between diabetics and non-diabetics in rates of second generation antipsychotic use, multiple antipsychotic medication use or chlorpromazine equivalent daily antipsychotic dosage. Based on these findings, medication terms were dropped from multivariable analyses. Following this, a binary logistic regression procedure was conducted to control for demographic variables of age, sex and race to examine the relationship between schizophrenia and diabetes. Higher age (P = 0.000) and non-Caucasian race (P = 0.000) was significantly predictive of diabetes status and male sex approached significance (P = 0.07). After controlling for these demographic factors, schizophrenia remained highly associated with diabetes with an odds

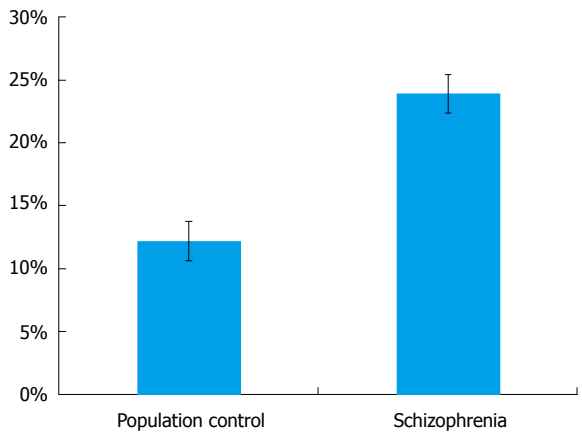

Figure 3 Diabetes mellitus prevalence in schizophrenia *vs* population (%).

ratio of 2.42 (95%CI: 1.75, 3.36, P = 0.000). Since obesity is closely associated with diabetes, one more regression analysis was performed including first BMI, and then obesity status (Table 2) and schizophrenia still remained highly significantly associated with diabetes.

DISCUSSION

In this study, patients with schizophrenia had a significantly higher average BMI, higher percentage of people with class 2 and class 3 obesity and higher percentage of people with diabetes, compared to the general population. The association for both obesity and diabetes remained after controlling for differences in demographic variables between the two groups. Having schizophrenia was associated with more than a 3-fold risk of obesity and more than a 2-fold risk of diabetes.

These results are consistent with what is known about the increased risk for metabolic syndrome in patients with schizophrenia. But a notable finding is that after controlling for BMI and obesity status, the risk of diabetes remained significant, though lower. Though pathways of diabetes development are thought to be the largely the same as those causing obesity, diabetes can occur without weight gain in patients on antipsychotic medications[14]. It is postulated that antipsychotics may to some extent affect glucose regulation independent of their effect on weight.

In this study design, medication variables for the general population were unknown and could not be compared with the schizophrenia group. Within the schizophrenia group, antipsychotic dose, second generation antipsychotics and use of multiple antipsychotic medications did not vary between the different categories of obesity or with diabetes status. It is notable that antipsychotic medication factors did not account for the differences in either obesity or diabetes status within the schizophrenia group. Neither the antipsychotic category nor the medication dose correlated with obesity. The strong risk of obesity and diabetes in schizophrenia coupled with lack of differential effects between antipsychotic medications and persistent risk

Table 2 Adjusted odds of diabetes mellitus in schizophrenia

	B	S.E.	Wald	Df	*P*	Odds ratio	95%CI for Odds ratio	
							Lower	Upper
Age	0.36	0.005	61.88	1	0	1.036	1.027	1.045
Sex (male)	0.34	0.133	6.51	1	0.011	1.405	1.082	1.825
Race (non-white)	0.52	0.156	11.29	1	0.001	1.689	1.224	2.292
Obesity	1.25	0.137	84.61	1	0	3.514	2.689	4.593
Schizophrenia	0.6	0.179	11.31	1	0.001	1.825	1.285	2.59

of diabetes after controlling for obesity in this sample indicates some of the risk may be due to factors other than medications. Many other factors including an innate risk may be responsible for the higher prevalence of obesity and diabetes in the schizophrenia population. An inherent susceptibility to diabetes in schizophrenia patients is supported by studies with medication naive first episode psychosis patients[10]. The inherent risk for diabetes may be mediated in part by elevated levels of inflammatory cytokines seen in both schizophrenia and obesity[9]. On the other hand, cross sectional data may not be sufficient to unravel the complicated relationship between schizophrenia, antipsychotic medications, obesity and diabetes since patients go through medication changes over the course of the disease.

Other factors may affect these results. The survey data from BRFSS is based on self-report by metropolitan area residents. People may have undiagnosed diabetes and tend to underestimate and underreport obesity. Patients with schizophrenia have increased contact with health care leading to higher likelihood of diabetes screening during mental health treatment. However, the increased prevalence of diabetes seen in this study is consistent with earlier reports and is unlikely to result from a screening bias given that historically people with SMI have low rates of screening and treatment for metabolic conditions[15].

This study did not find a correlation between second generation antipsychotics and obesity and diabetes status. It may be that the differential effects of first and second generation antipsychotics on metabolic syndrome are not as different as previously believed. Indeed, the rates of metabolic syndrome between first and second generation antipsychotics are not significant when clozapine and olanzapine are excluded[5]. A meta-analysis of antipsychotic-induced weight gain in first episode psychosis patients showed that most antipsychotic medications, including first generation medications like haloperidol, are associated with some weight gain[16].

An innate predisposition to diabetes, seen in first episode psychosis patients, may be compounded by antipsychotic medications, lower socioeconomic status and decreased access to quality health care. Patients with schizophrenia also are less physically active contributing further to insulin resistance. Antipsychotics alone may not account for the high metabolic burden seen in chronic schizophrenia patients and high mortality rates. In fact, antipsychotic use is associated with overall lower mortality, especially when highly effective medications like clozapine are used for treating schizophrenia[17].

Strengths of this study include the large naturalistic sampling of community dwelling schizophrenia patients as well as a local population control sample in the same geographic area. Patients were not recruited for the study, instead all patients in the Psychosis Program with diagnosis of schizophrenia were included. This study design allows for applicability of results to real world settings. In the community mental health center sample, schizophrenia diagnosis was based on a structured interview and diabetes diagnosis was established based on previous lab diagnosis. A limitation is that the BRFSS survey data was based on self-report. Also the antipsychotic use is cross sectional and results may be confounded by changes in the type of antipsychotic used throughout patients' disease history. Since this was not a controlled study, demographic variables were different between the disease and control groups. While these may confound results, in our study, higher prevalence of obesity and diabetes in schizophrenia persisted after controlling for these variables.

In conclusion, this study is consistent with previous research showing significantly increased prevalence of obesity and diabetes in people with schizophrenia. The risk for diabetes is present even when weight is controlled for as a causative factor. Antipsychotics contribute to the burden of diabetes but may not be the primary cause. Since schizophrenia patients are associated with a very high risk of diabetes, clinicians should be vigilant about screening and monitoring patients for diabetes from the beginning of treatment.

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COMMENTS

Background

People with serious mental illness (SMI), including schizophrenia, have

excess morbidity and mortality compared with the rest of the population, largely attributable to physical illness, including metabolic abnormalities and cardiovascular disease. The rates of obesity and diabetes in patients with schizophrenia are higher than the general population. The cause for the increased prevalence of these conditions is multifactorial. Antipsychotics, unhealthy diets, inadequate physical activity due to lower socioeconomic status, lower educational level, and sub-optimal living situations and symptoms such as low motivation, apathy and cognitive deficits could all play a role in increased metabolic risks in this population. An additional important contributing factor to the increased prevalence of diabetes in schizophrenia may be an inherent susceptibility to diabetes in people with schizophrenia. People with schizophrenia may have increased vulnerability manifesting as pre-diabetes that is compounded by cumulative exposure to antipsychotic medications. This study compares the prevalence of obesity and diabetes in schizophrenia patients treated at a community mental health center with controls in the same metropolitan area. The study also examines the effect of antipsychotic exposure on diabetes prevalence in schizophrenia patients.

Research frontiers

Many studies show there may be an inherent risk for diabetes in schizophrenia patients as evidenced by increased insulin resistance in patients with new onset of psychosis. Inflammatory pathways seen in both schizophrenia and people with obesity and diabetes may mediate some of this risk. The pathways leading to obesity and those leading to diabetes are not well differentiated. All these are important areas for further research. The detrimental effect of antipsychotics on weight and insulin resistance is well known. However, the extent of this risk compared to other factors is not clear. The contribution of various known risk factors on development of metabolic syndrome in people with schizophrenia is an area for further study.

Innovations and breakthroughs

More and more studies are establishing that there is a high prevalence of obesity and diabetes in schizophrenia. There is emerging evidence that some risk of diabetes may be present independent of weight. The present study adds to existing evidence that antipsychotics, while contributing to burden of diabetes, are not the only cause.

Applications

This naturalistic study is consistent with existing research that shows high prevalence of obesity and diabetes in schizophrenia patients. The risk of diabetes appears to be mediated not only by weight gain but other factors. Hence, not only weight but also diabetes screening should be prioritized in schizophrenia populations. Also this should be done regardless of whether they are on antipsychotic medications or not. Clinicians should be vigilant about early detection and treatment. Many studies in people after the first episode of psychosis demonstrate that early detection and treatment of obesity and diabetes may improve morbidity and mortality.

Terminology

First generation antipsychotics refer to a group of antipsychotics used to treat schizophrenia, whose primary mechanism of action is *via* dopamine. Second generation antipsychotics refer to antipsychotics that were developed later and have pharmacologic actions on both dopamine and serotonin. The latter group is generally considered to carry a higher risk for metabolic syndrome.

Peer-review

The manuscript is well written and concise.

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ORIGINAL ARTICLE

Observational Study

Age-dependent changes in the association between sleep duration and impaired glucose metabolism

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Author contributions: Nakajima K designed the overall study and analyzed the data; Suwa K identified eligible subjects from the database at Saitama Health Promotion Corporation and confirmed validation of the measurements and methods; Toyama K prepared the manuscript, including editing and discussion; Nakajima K wrote the manuscript and is the guarantor of the manuscript; all authors read and approved the final manuscript.

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Abstract

AIM

To investigate whether the association between sleep duration and impaired glucose metabolism varies among younger and older populations.

METHODS

We reviewed data of self-reported habitual sleep duration per night, HbA1c levels, and clinically relevant factors in a cross-sectional checkup database of 75472 Japanese from the general population aged 20-79 years (51695 men and 23777 women). Associations of prediabetes (HbA1c $\geqslant$ 5.7% and/or diabetic pharmacotherapy) or diabetes (HbA1c $\geqslant$ 6.5% and/or diabetic pharmacotherapy) with short and long sleep durations compared with a reference sleep duration (7 h) were investigated by multivariate logistic regression analysis. We controlled for potential relevant confounders, including age, sex, and work duration per day according to younger and older subjects.

RESULTS

As age advanced, sleep duration became longer and this increase in the 40s and 50s was two times greater in men than in women. This finding was accompanied by a deterioration in HbA1c levels. In subjects aged younger

than 40 years (n = 32929), HbA1c levels were inversely and linearly correlated with sleep duration in both sexes. However, in subjects aged 40 years or older (n = 42543), HbA1c levels showed a non-linear relationship against sleep duration with a nadir at 7 h. Multivariate logistic regression analysis showed that in younger subjects, short durations of sleep (≤ 5 h and 6 h) were positively associated with prediabetes (both P < 0.001), but a long duration of sleep (≥ 8 h) was inversely associated with prediabetes (P < 0.001). These associations remained significant after adjustment for relevant confounders, including age, sex, and work duration per day (ORs = 1.20, 95%CI: 1.05-1.37, P < 0.001; ORs = 1.12, 95%CI: 1.02-1.24, P < 0.05; and ORs = 0.84, 95%CI: 0.72-0.99, P < 0.05, respectively). In contrast, in older subjects, besides an association of prediabetes with a short duration of sleep (≤ 5 h) (ORs = 1.12, 95%CI: 1.03-1.21, P < 0.01), diabetes was significantly associated with a long duration of sleep (≥ 8 h) (ORs = 1.11, 95%CI: 1.02-1.25, P < 0.05).

CONCLUSION

A short sleep duration may be associated with prediabetes throughout life. However, the association between a long sleep duration and glucose metabolism can change with aging.

Key words: Sleep; Prediabetes; Diabetes; HbA1c; Aging

Core tip: Short and long durations of sleep have been putatively associated with type 2 diabetes. However, whether age affects these associations is unknown, although sleep duration and glucose homeostasis can change with advancing age. Our study demonstrated that a short sleep duration may be associated with prediabetes throughout the lifespan, whereas a long duration of sleep may be inversely associated with prediabetes in younger subjects. Additionally, a long sleep duration was associated with diabetes in older subjects. Therefore, aging may substantially affect the association between a long sleep duration and glucose homeostasis.

Nakajima K, Suwa K, Toyama K. Age-dependent changes in the association between sleep duration and impaired glucose metabolism. *World J Diabetes* 2017; 8(8): 397-406 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i8/397.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i8.397

INTRODUCTION

For the last two decades, many clinical studies have shown that shorter and longer durations of sleep are associated with health hazards, including type 2 diabetes, metabolic syndrome, and increased all-cause mortality[1-16]. Although a short sleep duration may be robustly associated with impaired glucose homeostasis, there are conflicting results, especially concerning a long sleep duration[3,5,8,13,15]. Generally, glucose homeostasis is aggravated with aging, probably owing to reduced pancreatic β-cell function and increased insulin resistance[17-19]. In contrast, individual's sleep duration can become longer and its quality can be aggravated (*e.g.*, more fragmented) as people become older[11,20], although it has been shown that objectively measured sleep duration generally decreases with age[21]. Taken together, these findings suggest that glucose homeostasis and sleep duration likely change with advancing age. However, to date, the effect of aging on the association between sleep duration and impaired glucose metabolism is less clear, regardless of accumulated evidence[1-7,9-15].

Based on the findings mentioned above and the worldwide extension of the life span[22,23], we investigated whether the association between self-reported sleep duration and dysglycemia varies among younger and older generations in the general Japanese population who undergo an annual medical checkup.

MATERIALS AND METHODS

This cross-sectional study consisted of data that were recorded in medical checkups of people living or working in Saitama, a suburb of Tokyo, Japan. The original study has been described in more detail elsewhere[24]. The current study involved two institutions in Kanagawa and Saitama, Japan, including Kanagawa University of Human Services and Saitama Health Promotion Corporation, a public interest corporation. The protocol was approved by the Ethics Committee of Kanagawa University of Human Services (No. 10-22). All procedures that were followed were in accordance with the ethical standards of the responsible committee on human experimentation (Kanagawa University of Human Services, Japan) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study.

Subjects

We reviewed the data for 116817 subjects who underwent a medical health checkup at the Saitama Health Promotion Corporation in 2007. Individuals who required immediate treatment for serious conditions, such as suspected cancer, heart failure, atherosclerotic disease, or infectious pneumonia, were not included from the beginning of the study. All recruited subjects, who were free from disability and hemiplegia, answered a questionnaire about their lifestyle characteristics. After exclusion of subjects with incomplete data (n = 41345), 75472 apparently healthy subjects aged 20-79 years were enrolled, without any special selections. Subjects were primarily divided into younger subjects (< 40 years old, n = 32929) and older subjects (≥ 40 years old, n = 42543).

Anthropometric and laboratory tests, and sleep duration

All anthropometric and laboratory tests were carried out in the morning. Body mass index was calculated as weight (kg) divided by height (m^2). Serum parameters were measured using standard methods by the Hitachi autoanalyzer (Tokyo, Japan) at Saitama Health Promotion Corporation. Glycated hemoglobin (HbA1c) was measured in Japan Diabetes Society (JDS) HbA1c units, which were converted to National Glycohemoglobin Standardization Program HbA1c units using the officially certified formula: HbA1c (NGSP) (%) = 1.02 × JDS (%) + 0.25%[25].

Prediabetes (including diabetes) was defined as HbA1c ⩾ 5.7% or any pharmacotherapy for diabetes. Diabetes was defined as HbA1c ⩾ 6.5% or any pharmacotherapy for diabetes[26]. Accordingly, subjects with prediabetes included those with diabetes. We considered the white blood cell (WBC) count, a surrogate marker for inflammation, as an important confounding factor for the association between sleep duration and dysglycemia. However, available data of the WBC count were limited in this study (n = 74837). Self-reported sleep duration per night, which was obtained as a response to a simple question about sleep, was divided into five categories of ⩽ 5, 6, 7, 8, and ⩾ 9 h of sleep duration.

Statistical analysis

Data are expressed as the mean ± SD or median (interquartile range). Differences in continuous variables between men and women were assessed by the *t*-test. In each age group, subjects were divided into four groups according to sleep duration per night: ⩽ 5, 6, 7, and ⩾ 8 h. The percentage of subjects with ⩾ 9 h sleep duration was small (1.1%). Therefore, subjects with an 8-h sleep duration and subjects with ⩾ 9 h of sleep duration were grouped together as subjects with ⩾ 8 h of sleep duration. *P* values for continuous variables were determined using ANOVA and for categorical variables using the χ^2 test. Linear correlations were examined by Pearson's correlation coefficients after coding ⩽ 5, 6, 7, 8, and ⩾ 9 h of sleep duration as continuous values of 5-9, respectively. Multivariate logistic regression models were used to examine the associations between sleep duration and prediabetes or diabetes, compared with a reference sleep duration of 7 h[5,7,8]. These models were used with or without adjustment for relevant confounders, which yielded crude and adjusted odds ratios and 95%CIs. Tests for linear trends (*P* for trend) were calculated by treating sleep duration as a continuous variable (*i.e.*, 1-4 for a sleep duration of ⩽ 5, 6, 7, and ⩾ 8 h, respectively), and the same model analysis was conducted. Statistical review of the study was performed by Dr. Eiichiro Kanda, MD, PhD, MPH, Department of Nephrology, Tokyo Kyosai Hospital, Tokyo, Japan. Statistical analyses were performed using SPSS software version 22.0 (SPSS-IBM, Chicago, IL, United States) and Statview version 5.0 (SAS Institute, Cary, NC, United States). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Clinical characteristics of subjects in the younger and older groups are shown in Tables 1 and 2, respectively. Overall, in younger and older subjects, as sleep duration increased, age increased and work duration decreased (both $P < 0.0001$, ANOVA). In older subjects, as sleep duration became longer, most of the parameters became worse (all $P < 0.0001$), except for body mass index (BMI) and the prevalence of regular exercise. In short-duration sleepers, the prevalence of current smokers was higher in younger subjects, whereas it was lower in older subjects. Notably, the rates of current smokers and everyday alcohol drinkers were prevalent in older long sleepers (41.1% and 32.0%, respectively) among the overall subjects (Table 2). Duration of sleep was inversely correlated with the WBC count in younger subjects (r = -0.03, $P < 0.0001$, Pearson's correlation), but it was positively correlated with the WBC count in older subjects (r = 0.02, $P < 0.0001$).

Figure 1 shows overall sleep duration according to age groups and sex. Sleep duration became prolonged as age advanced, and this increase was approximately two times greater in men than in women at middle age (40-59 years) owing to a dip in sleep duration in women. HbA1c levels increased with increasing age in both sexes (Figure 2). In younger subjects, sleep duration was inversely and linearly correlated with HbA1c levels (r = -0.04, $P < 0.0001$), regardless of sex (Figure 3). In older subjects, HbA1c levels showed a non-linear relationship against sleep duration with a nadir 7 h. When subjects were divided by every 10 years, similar results were observed (Figure 4), but the relationship of HbA1c was almost flat against sleep duration in subjects in their 40s (r = -0.006, P = 0.41).

Multivariate logistic regression analysis showed that in younger subjects, short durations of sleep (⩽ 5 h, 6 h) were positively associated with prediabetes compared with the reference duration of sleep (7 h). These findings remained significant after adjustment for relevant confounders (Table 3) ($P < 0.01$ and $P < 0.05$, respectively). In contrast, a long duration of sleep (⩾ 8 h) was inversely associated with prediabetes ($P < 0.05$). Either short or long duration of sleep were not significantly associated with diabetes after full adjustment for relevant confounding factors. In older subjects, a short duration of sleep (⩽ 5 h) was positively associated with prediabetes, which also remained significant after full adjustment (Table 4) ($P < 0.01$). However, a long duration of sleep (⩾ 8 h) was marginally associated with diabetes after full adjustment for relevant confounders ($P < 0.05$). Overall, prediabetes was inversely associated with sleep duration in younger and older subjects ($P < 0.001$ and $P < 0.01$ for linear trend, respectively). However,

Table 1 Characteristics of younger subjects classified by sleep duration

Sleep duration	⩽ 5 h	6 h	7 h	⩾ 8 h	*P* values
n (%)	4110 (12.5)	16265 (49.4)	9377 (28.5)	3177 (9.6)	
Male, *n* (%)	2865 (69.7)	11121 (68.4)	6349 (67.7)	2026 (63.8)	< 0.0001
Age (yr)	30.5 ± 5.5	30.4 ± 5.3	30.7 ± 5.3	31.1 ± 5.3	< 0.0001
BMI (kg/m^2)	23.4 ± 3.9	23.1 ± 3.7	22.8 ± 3.6	23.0 ± 3.8	< 0.0001
Systolic blood pressure (mmHg)	118 ± 14.3	118 ± 14.5	118 ± 14.6	119 ± 14.8	0.58
White blood cell count (× 10^9/L)	6.67 ± 1.8	6.48 ± 1.7	6.46 ± 1.7	6.48 ± 1.9	< 0.0001
Serum triglycerides (mmol/L)	0.9 (0.6-1.5)	0.9 (0.6-1.4)	0.9 (0.6-1.5)	0.9 (0.6-1.5)	0.46
Serum HDL-cholesterol (mmol/L)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4	0.04
HbA1c (NGSP, %)	5.35 ± 0.5	5.32 ± 0.5	5.30 ± 0.4	5.29 ± 0.5	< 0.0001
Work duration (h/d)	9.4 ± 1.5	9.0 ± 1.4	8.6 ± 1.3	8.1 ± 1.4	< 0.0001
Pharmacotherapy for					
Hypertension, *n* (%)	25 (0.6)	73 (0.4)	38 (0.4)	19 (0.6)	0.29
Diabetes, *n* (%)	13 (0.3)	39 (0.2)	22 (0.2)	11 (0.3)	0.83
Dyslipidemia, *n* (%)	11 (0.3)	46 (0.3)	24 (0.3)	22 (0.7)	0.0001
Current smokers, *n* (%)	1791 (43.6)	5980 (36.8)	3221 (34.4)	964 (30.3)	< 0.0001
Everyday alcohol consumers, *n* (%)	353 (8.6)	1386 (8.5)	1022 (10.9)	370 (11.6)	< 0.0001
Regular exercisers, *n* (%)[1]	553 (14.4)	3154 (17.2)	2740 (19.4)	1367 (21.8)	< 0.0001
Past history of					
CVD, *n* (%)	46 (1.1)	129 (0.8)	102 (1.1)	46 (1.4)	0.002

Data are presented as mean ± SD, median (interquartile range), or *n* (%). *P* values were determined by ANOVA and χ^2 tests were used for continuous and categorical variables. Serum triglyceride concentrations were log transformed before parametric analysis. [1]Regular exercise was defined as ⩾ 30 min exercise per session at least twice a week. Values of white blood cell count are only available in 32713 subjects (*n* = 4073, 16171, 9313, and 3156, for ⩽ 5 h, 6 h, 7 h, and ⩾ 8 h sleep duration, respectively). BMI: Body mass index; HDL: High-density lipoprotein; NGSP: National Glycohemoglobin Standardization Program; CVD: Cardiovascular disease.

Table 2 Characteristics of older subjects classified by sleep duration

Sleep duration	⩽ 5 h	6 h	7 h	⩾ 8 h
n (%)	3838 (9.0)	18319 (43.1)	14115 (33.2)	6271 (14.7)
Male, *n* (%)	2197 (57.2)	11630 (63.5)	10399 (73.7)	5108 (81.5)
Age (yr)	49.4 ± 6.9	50.4 ± 6.8	51.8 ± 7.2	54.3 ± 8.4
BMI (kg/m²)	23.9 ± 3.7	23.8 ± 3.5	23.7 ± 3.3	23.7 ± 3.2
Systolic blood pressure (mmHg)	125 ± 18.2	126 ± 18.2	128 ± 18.4	131 ± 19.0
White blood cell count (× 10^9/L)	6.6 ± 1.8	6.5 ± 1.7	6.5 ± 1.7	6.6 ± 1.8
Serum triglycerides (mmol/L)	1.1 (0.7-1.8)	1.2 (0.8-1.8)	1.3 (0.8-1.9)	1.3 (0.9-2.0)
Serum HDL-cholesterol (mmol/L)	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4
HbA1c (NGSP, %)	5.69 ± 0.8	5.69 ± 0.8	5.70 ± 0.8	5.77 ± 0.9
Work duration (h/d)	8.9 ± 1.7	8.5 ± 1.5	8.3 ± 1.3	8.1 ± 1.3
Pharmacotherapy for				
Hypertension, *n* (%)	355 (9.3)	1970 (10.8)	1843 (13.1)	1091 (17.4)
Diabetes, *n* (%)	138 (3.6)	633 (3.5)	559 (4.0)	343 (5.5)
Dyslipidemia, *n* (%)	109 (2.8)	621 (3.4)	536 (3.8)	278 (4.4)
Current smokers, *n* (%)	1239 (32.3)	5981 (32.6)	4982 (35.3)	2580 (41.1)
Everyday alcohol consumers, *n* (%)	651 (17.0)	3378 (18.4)	3493 (24.7)	2009 (32.0)
Regular exercisers, *n* (%)[1]	611 (14.9)	2938 (18.1)	1913 (20.4)	617 (19.4)
Past history of				
CVD, *n* (%)	125 (3.3)	620 (3.4)	568 (4.0)	323 (5.2)

Data are presented as mean ± SD, median (interquartile range), or *n* (%). *P* values determined by ANOVA and χ^2 tests for all continuous and categorical variables listed above were < 0.0001 (data not shown). Serum triglyceride concentrations were log transformed before parametric analysis. [1]Regular exercise was defined as ⩾ 30 min exercise per session at least twice a week. Values of white blood cell count are only available in 42124 subjects (*n* = 3809, 18172, 13973, and 6170, for ⩽ 5 h, 6 h, 7 h, and ⩾ 8 h sleep duration, respectively). BMI: Body mass index; HDL: High-density lipoprotein; NGSP: National Glycohemoglobin Standardization Program; CVD: Cardiovascular disease.

a significant association was not observed between diabetes and duration of sleep in both generations.

DISCUSSION

This large, epidemiological study of the general Japanese population showed that a short duration of sleep was robustly associated with prediabetes, but not diabetes, in young and old generations. In contrast, a long duration of sleep was inversely associated with prediabetes in the young generation, but it was positively associated with diabetes in the old generation. Therefore, aging may affect the relationship between a long sleep duration and glucose homeostasis.

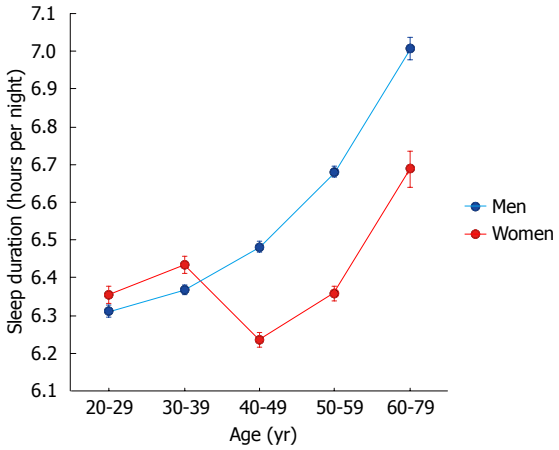

Figure 1 Sleep duration according to age groups and sex. Each point and vertical bar represent the mean ± 1.96 SE. Sleep duration in men and women increased with increasing age (both $P < 0.0001$, ANOVA). Significant differences were observed in sleep duration between men and women in all age groups (all $P < 0.0001$, except for $P = 0.003$ in the 20s, *t*-test). The corresponding number of subjects is shown in the side of the bar.

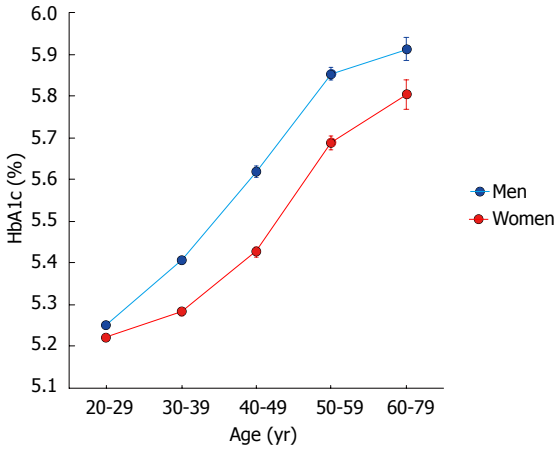

Figure 2 HbA1c levels according to age groups divided by decades. Each point and vertical bar represent the mean ± 1.96 SE. HbA1c levels in men and women increased with increasing age (both $P < 0.0001$, ANOVA). Significant differences were observed in HbA1c levels between men and women in all age groups (all $P < 0.0001$, *t*-test). The corresponding number of subjects is the same as that in Figure 1.

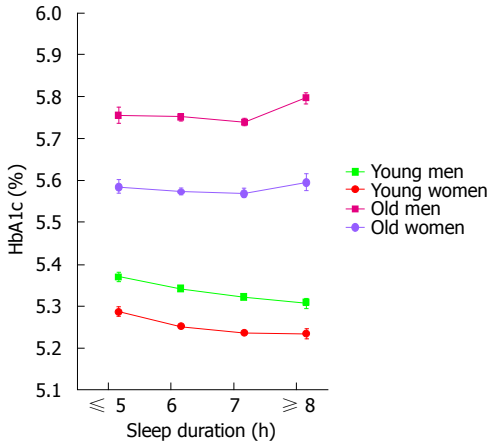

Figure 3 Relationship between HbA1c levels and sleep duration according to age and sex. Each point and vertical bar represent the mean ± SE. *P* values for ANOVA were < 0.0001, 0.0001, 0.002, and 0.56 for young men, young women, older men, and older women, respectively. The corresponding number of subjects is shown in the side of the bar.

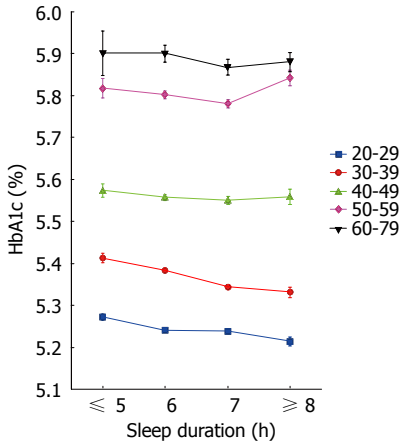

Figure 4 Relationship between HbA1c levels and sleep duration according to age groups divided by decades. Each point and vertical bar represent the mean ± SE. Correlation coefficients and *P* values of Pearson's correlation were $r = -0.03$, $P < 0.0001$ for the 20s, $r = -0.05$, $P < 0.0001$ for the 30s, $r = -0.01$, $P = 0.41$ for the 40s, $r = 0.01$, $P = 0.52$ for the 50s, and $r = -0.01$, $P = 0.32$ for the 60-70s, respectively. The corresponding number of subjects is shown in the side of the bar.

A significant association between a short sleep duration and prediabetes is consistent with many previous studies[1,3,5,6-13,15,16]. This association could be explained by physiological mechanisms, such as insulin resistance[27,28], decreased leptin levels, increased ghrelin levels and inflammation, sympathetic nervous system activation, and oxidative stress[29]. This association could also be explained by behavioral mechanisms, such as increased food intake, and unfavorable lifestyles, such as smoking and sedentary behavior[28]. Long-lasting wakefulness and arousal can increase the level of orexin, a hypothalamic neuropeptide, which is found in the brain and stimulates appetite and food intake[30-32]. Additionally, a short sleep duration can be associated with abnormal eating behavior around sleep (AEBAS), such as breakfast skipping and/or late-night-dinner eating. AEBAS is often observed in younger people[33]. Sleep duration was shorter in younger subjects in our study. Although data for AEBAS was lacking in our study, previous studies have shown that AEBAS is associated with metabolic syndrome and hyperglycemia[33,34]. An elevated WBC and BMI, a higher prevalence of current smokers, and a lower prevalence of regular exercisers in young short-duration sleepers compared with young long-duration sleepers (Table 1) may be compatible with these explanations.

The most plausible explanation for the null association between a short duration of sleep and diabetes, instead of prediabetes, may be partially due to an insufficient number of cases of overt diabetes.

Table 3 Odds ratios (95%CIs) of sleep duration for prediabetes and diabetes in younger subjects *n* (%)

Sleep durations	⩽ 5 h	6 h	7 h	⩾ 8 h	*P* value
Prediabetes					
Cases	418 (10.2)	1463 (9.0)	743 (7.9)	229 (7.2)	
Model 1	1.32 (1.16-1.49)[d]	1.15 (1.05-1.26)[b]	1 (reference)	0.90 (0.77-1.05)	0.88 (0.84-0.92)[d]
Model 2	1.32 (1.16-1.50)[d]	1.18 (1.07-1.29)[b]	1 (reference)	0.89 (0.76-1.04)	0.87 (0.83-0.91)[d]
Model 3	1.20 (1.05-1.37)[b]	1.12 (1.02-1.24)[a]	1 (reference)	0.84 (0.72-0.99)[a]	0.90 (0.86-0.95)[d]
Diabetes					
Cases	58 (1.4)	150 (0.9)	77 (0.8)	27 (0.9)	
Model 1	1.73 (1.23-2.44)[b]	1.12 (0.85-1.42)	1 (reference)	1.04 (0.67-1.61)	0.83 (0.72-0.96)[a]
Model 2	1.72 (1.22-2.43)[b]	1.16 (0.88-1.52)	1 (reference)	1.01 (0.65-1.58)	0.83 (0.72-0.95)[b]
Model 3	1.35 (0.93-1.97)	1.02 (0.76-1.37)	1 (reference)	0.92 (0.58-1.46)	0.90 (0.78-1.04)

Model 1: Unadjusted; Model 2: Adjusted for age and sex; Model 3: Model 2 plus adjustment for current smoking (*vs* non-smoking), daily alcohol consumption (*vs* infrequent/no alcohol consumption), regular exercise (*vs* no regular exercise), pharmacotherapy (hypertension, diabetes, and dyslipidemia), BMI, WBC count, systolic blood pressure, triglycerides, HDL-cholesterol, duration of work (as continuous variables), and past history of CVD (*vs* no history). Triglyceride concentrations were log transformed before analysis. Regular exercise was defined as ⩾ 30 min exercise per session at least twice a week. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$. BMI: Body mass index; HDL: High-density lipoprotein; CVD: Cardiovascular disease; WBC: White blood cell.

Table 4 Odds ratios (95%CIs) of sleep duration for prediabetes and diabetes in older subjects *n* (%)

Sleep duration	⩽ 5 h	6 h	7 h	⩾ 8 h	*P* value
Prediabetes					
Cases	1259 (32.8)	6006 (32.8)	4788 (33.9)	2343 (37.4)	
Model 1	0.95 (0.88-1.03)	0.95 (0.91-0.995)[a]	1 (reference)	1.16 (1.09-1.24)[d]	1.07 (1.05-1.10)[d]
Model 2	1.17 (1.09-1.27)[d]	1.08 (1.03-1.14)[b]	1 (reference)	0.97 (0.91-1.03)	0.94 (0.91-0.96)[d]
Model 3	1.12 (1.03-1.21)[b]	1.04 (0.99-1.11)	1 (reference)	0.97 (0.91-1.04)	0.96 (0.94-0.99)[b]
Diabetes					
Cases	271 (7.1)	1379 (7.5)	1181 (8.4)	702 (11.2)	
Model 1	0.83 (0.73-0.95)[b]	0.89 (0.82-0.97)[b]	1 (reference)	1.38 (1.25-1.52)[d]	1.19 (1.15-1.24)[d]
Model 2	1.10 (0.95-1.26)	1.06 (0.98-1.15)	1 (reference)	1.11 (1.00-1.22)	1.00 (0.96-1.04)
Model 3	1.02 (0.88-1.18)	1.01 (0.93-1.11)	1 (reference)	1.11 (1.02-1.25)[a]	1.03 (0.99-1.08)

Model 1: Unadjusted; Model 2: Adjusted for age and sex; Model 3: Model 2 plus adjustment for current smoking (*vs* non-smoking), daily alcohol consumption (*vs* infrequent/no alcohol consumption), regular exercise (*vs* no regular exercise), pharmacotherapy (hypertension, diabetes and dyslipidemia), BMI, WBC count, systolic blood pressure, triglycerides, HDL-cholesterol, duration of work (as continuous variables), and past history of CVD (*vs* no history). Triglyceride concentrations were log transformed before analysis. Regular exercise was defined as ⩾ 30 min exercise per session at least twice a week. [a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$. BMI: Body mass index; HDL: High-density lipoprotein; CVD: Cardiovascular disease; WBC: White blood cell.

This occurred because subjects were apparently healthy people who underwent a health screening checkup. Therefore, patients with poor glucose control were unlikely to be enrolled in this study. Before full adjustment for relevant confounding factors, a significant association between a short sleep duration and diabetes was observed with adjustment for age and sex in young subjects (Table 3). However, statistical significance of this association disappeared after full adjustment. This suggests that factors other than age and sex might contribute to the association between poor glucose metabolism and a short sleep duration. Indeed, patients with type 1 diabetes were not excluded in our study. However, most of the subjects who were determined as having diabetes were likely to have type 2 diabetes because of its higher prevalence in the general population (90%-95%)[26].

The reason for the discrepancy in the association between a long sleep duration and glucose metabolism among younger and older generations is unknown. Several studies have shown that a long duration of sleep may reflect underlying inflammatory etiologies in older people[4,35,36]. Pérez de Heredia *et al*[37] showed that sleep duration was negatively associated with the WBC count in adolescents. Consistent with this previous finding, in our study, the duration of sleep was inversely associated with the WBC count in younger subjects, whereas it was positively associated with the WBC count in older subjects. Taken together, in the young generation, a long sleep duration can provide sufficient rest and lead to improvement of metabolic homeostasis and inflammatory status. However, in older people, a long duration of sleep may reflect required long rest because of latent or overt disease[4,12,20]. This situation could simultaneously aggravate glucose homeostasis. Notably, in our study, the relationship between HbA1c levels and the duration of sleep appeared to be flat in subjects in their 40s, and a J-curve relationship gradually occurred after the 40s (Figure 4). This finding indicates that the etiological relation between a long sleep duration and

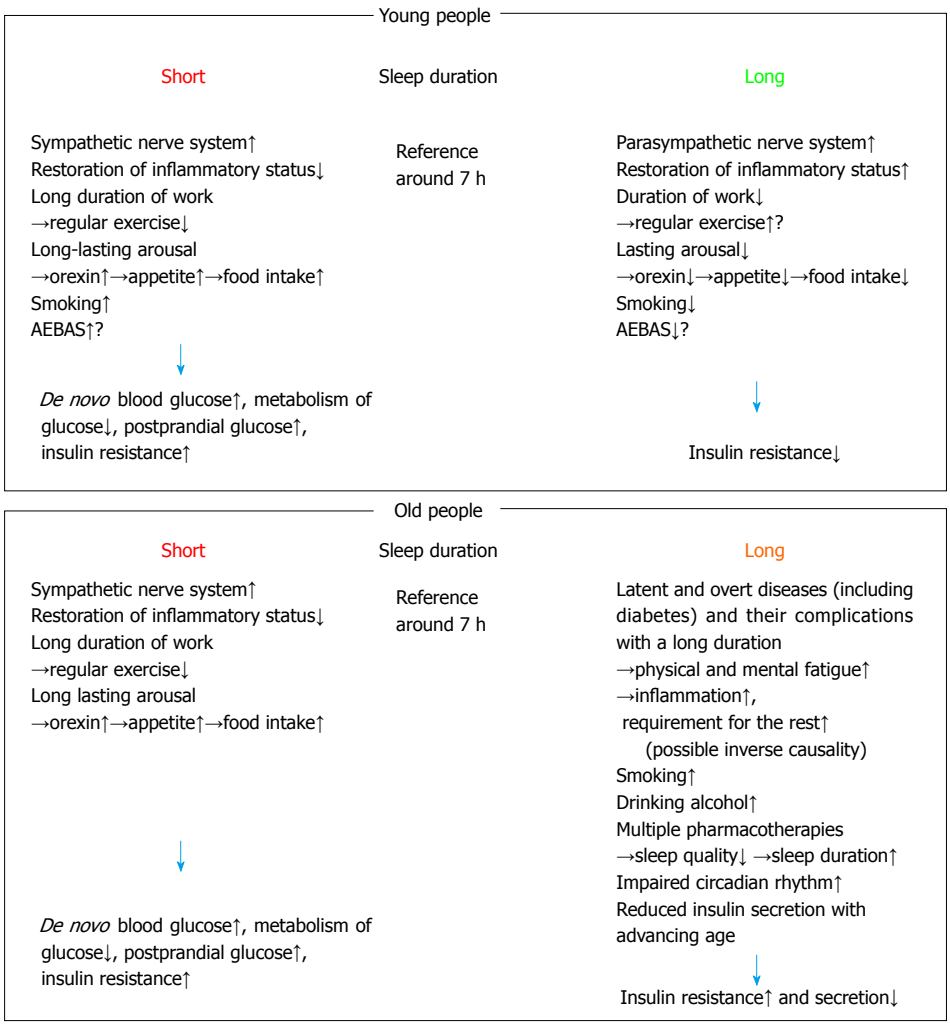

Figure 5 Relationship between sleep duration and impaired glucose metabolism, and plausible underlying mechanisms. AEBAS: Abnormal eating behavior around sleep.

impaired glucose homeostasis may occur approximately in the 40s. Mizukami *et al*[38] studied age-related changes of islet structure in Japanese non-diabetic subjects and showed that the mass of pancreatic β-cells was increased during maturation and slowly decreased after the 40s. Additionally, Uehara *et al*[39] reported in a large-scale Japanese working population that the prevalence of glucose abnormalities increased with advancing age, especially during the mid-40s and 50s. These findings may partly relate to our results regarding the relationship between long sleep duration, prediabetes, and diabetes. Alternatively, an increase in sleep duration rather than a long sleep duration *per se* may be a crucial factor that contributes to development of type 2 diabetes[40]. Among the parameters that were investigated in this study, the duration of work, which was longer in younger than in older subjects in this study, may be a pivotal environmental factor that could restrict the duration of sleep. Therefore, in terms of public health, caution should be exercised in people with a long work duration for preventing a short sleep duration, cardiometabolic disease, and other unfavorable lifestyles.

Currently observed relationships between sleep duration and impaired glucose metabolism in young and older people are summarized in Figure 5. Plausible underlying mechanisms are also described in Figure 5.

Several limitations should be mentioned in this study. First, this study was cross-sectional in nature, and did not allow us to determine the causality between abnormal sleep duration and impaired glucose metabolism. However, the age of subjects in the current study widely varied (20-79 years), which could reflect overall trajectories in sleep duration and glucose homeostasis in the general Japanese population. Second, assessment of sleep duration was self-reported and the quality of sleep was not investigated. In particular, in older people, the time spent in bed can be misinterpreted as sleep duration. Additionally, sleep may be fragmented[20] and actual sleep duration may be less than expected. Therefore, more detailed study is required to confirm the association between sleep duration and metabolic abnormalities, including type 2 diabetes. Third, whether prediabetes with a short sleep duration could lead to diabetes after a certain period during the lifetime is unclear. Long-term, prospective

studies are required to determine this issue. Finally, our study consisted of apparently healthy subjects who underwent an ordinary checkup. As people get older, they usually have more complications and chronic diseases, including cognitive impairment and mental disorders, such as depression. These etiologies often require some pharmacotherapies that predispose to disturbing homeostasis of sleep[41-43]. Prescription for insomnia increases as age advances, which also alters the sleep circadian rhythm[44,45]. Additionally, chronic use of hypnotic might aggravate glucose metabolism, although a conflicting result has been reported[46]. Unfortunately, such pharmacotherapy and sleep medication were not investigated in this study. Therefore, the current findings may not be applicable to other populations who have a different longevity and higher proportions of diabetes and comorbidities.

In conclusion, the current study shows that a short sleep duration may be associated with prediabetes throughout the lifetime, whereas a long sleep duration is inversely associated with prediabetes in younger people. This finding indicates that a long sleep duration leads to better glucose homeostasis. In contrast, a long sleep duration may be associated with diabetes in older people, which might reflect an inverse causality owing to chronic diseases and complications of diabetes. Therefore, aging may be a pivotal factor that affects the association between a long sleep duration and impaired glucose homeostasis. Further large studies are required to confirm the current findings and determine the underlying mechanism(s).

COMMENTS

Background

Many clinical studies have shown that shorter and longer durations of sleep are associated with cardiometabolic diseases including type 2 diabetes and metabolic syndrome. However, there are conflicting results, especially concerning a long sleep duration, albeit a short sleep duration may be robustly associated with impaired glucose homeostasis.

Research frontiers

Although it has been shown that sleep duration generally decreases with age, individual's sleep duration can become longer in the elderly. Therefore, comparison between young and old populations using the same methods and criteria may be important for the research investigating the association between sleep duration and metabolic disease.

Innovations and breakthroughs

The authors investigated the association between sleep duration and impaired glucose metabolism (prediabetes and diabetes) in a large epidemiological study consisting of 75472 apparently healthy subjects with a wide range of age (20-79 years old), which was subdivided into two age groups (young subjects less than 40 years and old subjects aged 40 years or older). In most of previous studies, subjects were limited to patients with type 2 diabetes, middle-aged, or the elderly. By contrast, the authors compared the results of analysis in two age groups, which include not only healthy subjects but also those with diabetes.

Applications

The cause-effect relationship between long sleep duration and impaired glucose metabolism can vary between young and old populations. In brief, long sleep duration may be a cause for the prevention of diabetes in young people, whereas it may be a result that originated from cardiometabolic diseases and their complications. Therefore, when one encounters a patient with diabetes concomitant with long sleep duration, the causality, backgrounds, and confounding factors should be carefully taken into consideration.

Terminology

In this article, the authors use the term, "abnormal eating behavior around sleep (AEBAS)", which the authors made first time based on the current and previous their studies. AEBAS may include overeating at dinner, late-night-dinner eating, eating snack after dinner, skipping breakfast, and their combinations. Such AEBAS can affect the duration of sleep and deteriorate the quality. On the contrary, abnormal durations of sleep likely deteriorate eating behaviors around sleep. The definition of "prediabetes" includes diabetes in this study, which may complicate the relationship between prediabetes and diabetes. It may be appropriate to use the term such as "hyperglycemia" instead of "prediabetes". However, in comparison with diabetes, the authors dared to use the term "prediabetes". Considering these, the current results should be interpreted with care.

Peer-review

The author's purpose of the investigation is very interesting, also for scientists from related research fields.

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ORIGINAL ARTICLE

Observational Study

Clinico-epidemiological factors of health related quality of life among people with type 2 diabetes

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Abstract

AIM

To investigate the quality of life (QOL) and its clinical and epidemiological correlates among people with type 2 diabetes.

METHODS

This cross-sectional study was conducted in Tabriz, Northwest of Iran, including a total of 394 people with type 2 diabetes using convenient sampling method from November 2014 to March 2015. General information including demographic, socioeconomic status and life-style factors were collected by trained interviewers. Clinical information was retrieved from clinic's record and QOL was assessed using the 26-item WHOQOL-BRIFE questionnaire. Univariate and multivariate linear regression were performed to assess the related factors and QOL dimensions.

RESULTS

The mean of overall health related QOL was 52.11 ± 11.53 and the maximum and minimum dimensions were

respectively seen in psychological (60.38 ± 14.54) and social (38.32 ± 16.94) dimensions. The results of multiple linear regression showed a significant overall relationship between HRQOL and age (*b* = -1.48%, 95%CI: -0.03 and -2.93) level of education (*b* = 4.12%, 95%CI: 2.73 and 5.5), number of comorbidities (*b* = -2.41%, 95%CI: -3.89 and -9.41), and level of income (*b* = 1.98, 95%CI: 0.05 and 3.9), functional limitation (*b* = -3.59, 95%CI: -2.26 and -4.92) and psychological distress (*b* = -2.02%, 95%CI: -2.83 and -1.21). Level of education, functional limitation, psychological distress were associated with the score of physical, mental and environmental dimensions, and number of comorbidities was associated with the score of physical and mental dimensions.

CONCLUSION

Based on our findings, lifestyle modification and increasing facilities of clinics providing service can be effective steps to improve the QOL among people with type 2 diabetes.

Key words: Diabetes mellitus; Type 2; Lifestyle; Quality of life; Psychological distress

Core tip: Health related quality of life (HRQOL) is an important outcome measure in chronic diseases. The aim of this study was to assess quality of life and a range of epidemiological and clinical factors among people with type 2 diabetes. The findings of the present study showed that age, level of education, income, body mass index, functional limitation, psychological distress and number of comorbidities have a decisive role on HRQOL of patients with type 2 diabetes. So, it is important to improve the HRQOL by considering above predictors as an appropriate mechanism for public health interventions for type 2 diabetes.

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INTRODUCTION

Diabetes is one of the most common metabolic diseases with increasing prevalence that reduces life expectancy by one third. Diabetes is known as a "silent epidemic" which due to the aging population, changing patterns of life, prevalence of risk behaviors and rapid growth of urbanization has increased around the world[1-3]. It is estimated that 415 million people worldwide and 4.5 million people in Iran had diabetes in 2015. It is predicted that the number rises to more than 642 million worldwide and 4.8 million in Iran by 2040. In addition diabetes caused 4.9 million deaths in 2014 and 48% of deaths occurred in people less than 60 years[4-6].

One of the important issues in the care of chronic diseases such as diabetes is to investigate the quality of their life, which significantly affects one's physical-psychological performance and social communication[7]. As defined by the World Health Organization, quality of life (QOL) refers to "individuals' perception of their position in life in terms of culture, value system where they live, goals, expectations, standards and priorities"[8,9]. In other words, the health related QOL (HRQOL) is a subjective issue that is measured using different dimensions include physical, mental and social functions[10]. HRQOL as a multi-dimensional concept focuses on the impact of health on QOL[11].

There is a mutual relationship between the quality of diabetes care and QOL so that reducing the HRQOL of people with type 2 diabetes leads to poor glycemic control and an increased risk of disease complications. On the other hand, poor quality of care leads to reduced HRQOL[12,13].

Some studies showed that demographic factors, socio-economic status, presence of comorbid conditions, and diabetes control affect HRQOL among people with type 2 diabetes. Results of most studies on this group of patients showed that their HRQOL was not desirable[14-18]. Considering that East Azerbaijan province, is among provinces, in which diabetes is highly prevalent and this disease is among research priorities outlined in the province as well as the different climatic, socio-cultural conditions, lifestyle of the area and the low quality of diabetes care that has been shown in multiple studies[19,20], the present study was designed and implemented in order to investigate the factors affecting the HRQOL of diabetic patients referred to diabetes clinics in Tabriz.

MATERIALS AND METHODS

The present study was a cross-sectional study, which was conducted by trained interviewers on 394 patients with type 2 diabetes referred to diabetes clinics in Tabriz (Imam Reza and Sina Hospitals) in the form of face to face interviews using convenient sampling method from November 2014 to March 2015. Inclusion criteria included the willingness to cooperate and participate in the study, having diabetes type Ⅱ, age group above 25 years, having records of diabetes care in clinics of Tabriz (at least for a year), living in Tabriz and lack of specific (hemophilia, thalassemia, *etc*.) or debilitating diseases leading to hospitalization. Exclusion criteria included death, emigration, or any disability that prevents the provision of information by patients. Information required for the project was collected using a two-part questionnaire.

In the first part of the questionnaire, sociodemographic and clinical characteristics including age, sex, marital status, income, insurance status, education level, type of treatment (diet, oral medications, insulin), having comorbidities (hypertension, depression, kidney

Table 1 Demographic characteristics of diabetic people referring to diabetes clinics of Tabriz, 2015

Variable	Subgroups	*n* (%)
Age[1]	⩽ 49	85 (21.6)
	59-50	147 (37.3)
	⩾ 60	162 (41.1)
Gender	Male	134 (34)
	Female	260 (66)
Level education	Illiterate	143 (36.3)
	Primary school	149 (37.8)
	Secondary school and higher	102 (25.9)
Marital status	Single	45 (11.4)
	Married	349 (88.6)
Occupation	Employed	70 (17.8)
	Housekeeper	252 (63.9)
	Retired/other	72 (18.3)
Health insurance	Yes	378 (95.9)
	No	16 (4.1)
Household monthly income[2]	< 500	25 (6.3)
	1000-500	199 (50.5)
	> 1000	170 (43.1)
Smoking status	Yes	40 (10.2)
	No	354 (89.8)

[1]Mean and standard deviation: 56.67 ± 9.01; [2]Amounts are in 10000 Rials (1 USD equals to 33000 Islamic Republic of Iran's Rials).

disease, cardiovascular disease, cancer and other diseases) complications (retinopathy, neuropathy, nephropathy, cardiovascular complications), duration of diabetes, functional limitation, Kessler psychological distress (K10) and family history as well as anthropometric measures were collected. In the second part, the 26-item WHOQOL-BRIFE questionnaire was used. This questionnaire evaluates four broad areas, including physical health, psychological health, social relationships and environment. This questionnaire contained two questions on the assessment of the overall HRQOL and the level of self-perception of QOL. The 24 the next questions evaluate physical health (7 questions), mental health (6 questions), social relationships (3 questions) and environment (8 questions). The questionnaire was scored using Likert-5 point scale; *i.e.*, every question is assigned five answers (never, low, medium, high, very high), to each of which 1 to 5 points is assigned, respectively. The higher score in each of the dimensions reflects the better QOL. During analysis stage, those questionnaires, more than 20% of questions of which are remained unanswered (6 questions and more), were excluded, After calculating the raw score in each dimension, the scores can be converted and analyzed to 0-100 or 4-20 scale[21,22]. In this study, the 0-100 scale was used to analyze the results. The validity and reliability of the Persian version of the questionnaire, was determined by Nejat *et al*[23] in 2005.

Descriptive statistics [mean, standard deviation and frequency (percent)] was performed and test-*t*, Mann-Whitney, ANOVA, Kruskal Wallis were used and Welch test was employed to analyze the HRQOL according to demographic data and treatment options. Also, the multiple regression models were used to show the association between independent factors with dimensions of QOL. The level of significance of (P = 0.05) was considered in the present study. Data analysis was performed using SPSS 23.

This project was approved by Ethics Committee of Tabriz University of Medical Sciences (Ethic approval numberTBZMED.REC.2015.55). In addition, at the beginning of the study, informed consent was obtained in written forms from all of the participants.

RESULTS

The mean patient age was 56.67 ± 9.01 years. of the majority of participants (66%) were female, and married (88.6%), 36% were illiterate, most of them (96%) had health insurance and 56.8% of them had a monthly income of less than 10 million Rials, respectively. Smokers accounted for 10.2% of the participants and 48.7% of patients suffered complications, in 39.6% of whom the neuropathy was observed. A total of 74.1% of people had comorbidities, the most prevalent of which was high blood pressure (40.4%). A total of 56.9% of them used oral medicine and 55.3% of patients had a family history of diabetes (Table 1).

The mean of overall HRQOL was 52.11 ± 11.53 and the maximum and minimum dimensions of HRQOL were respectively seen in psychological 60.38 ± 14.54 and social dimension 38.32 ± 16.74 (Table 2).

A total of 79.8% of individuals had undesirable BMI (< 25) and HRQOL score was significantly lower in all HRQOL dimensions. The majority (63.5%) of individuals mentioned the disease duration of over 7 years. Also, the association between disease duration and QOL was statistically significant in all dimensions, except in social relations dimensions. HRQOL scores were low in all dimensions in people with functional limitation and those suffering from two or more comorbidities and patients with kidney disease had the lowest HRQOL score in all dimensions but in physical and mental dimensions. Blood biochemical indicators such as levels of HbA1c, cholesterol levels were not significant in each of HRQOL dimensions (P = 0.05) (Table 3).

The results of multiple linear regression showed a significant overall relationship between HRQOL and age (b = -1.48%, 95%CI: -0.03 and -2.93) level of education (b = 4.12%, 95%CI: 2.73 and 5.5), number of comorbidities (b = -2.41%, 95%CI: -3.89 and -9.41), and level of income (b = 1.98, 95%CI: 0.05 and 3.9), functional limitation (b = -3.59, 95%CI: -2.26 and -4.92) and psychological distress (b = -2.02%, 95%CI: -2.83 and -1.21). Also, there was association between the physical (level of education, BMI, functional limitation, psychological distress and number of comorbidities), social (age, level of education and functional limitation), mental (level of education and functional limitation, psychological distress and the number of comorbidities) and environmental dimensions (level of education, functional limitation,

Table 2 The status of different domains of health related quality of life according to the gender of diabetic people referring to diabetes clinics of Tabriz, 2015

HRQOL dimensions	Total		Male		Female		*P*-value
	Mean	SD	Mean	SD	Mean	SD	
Physical health	51.24	13.34	54.97	12.92	49.34	13.18	< 0.001
Psychological health	60.38	14.54	65.26	13.30	57.88	14.54	< 0.001
Social relationship	38.32	16.74	41.96	16.71	36.46	16.48	0.002
Environmental	58.48	10.48	59.64	11.13	57.88	10.10	0.115
Total HRQOL score	52.11	11.53	55.46	11.34	50.39	11.27	< 0.001

HRQOL: Health related quality of life.

Table 3 Different dimensions of health related quality of life according to the clinical aspects of diabetes among diabetic people referring to diabetes clinics of Tabriz, 2015

Variable	Subgroups	*n* (%)	Physical health	Social relationship	Environmental	Psychological health	Total HRQOL
Gender	Male	134 (34)	54.97 (12.92)	41.96 (16.71)	59.65 (11.14)	65.26 (13.30)	55.46 (11.34)
	Female	260 (66)	49.34 (13.18)	36.46 (16.48)	57.88 (10.11)	57.88 (14.54)	50.39 (11.27)
	P-value	-	< 0.001	0.002	0.115	< 0.001	< 0.001
Age	≤ 49	85 (21.6)	58.65 (11.64)	47.8 (17.59)	61.64 (11.14)	64.11 (16.15)	58.8 (11.66)
	59-50	147 (37.3)	52.36 (12.97)	39.68 (15.17)	59.71 (10.9)	61.37 (14.71)	53.28 (11.19)
	≥ 60	162 (41.1)	46.32 (12.53)	32.08 (15.02)	55.68 (9.01)	57.5 (12.91)	47.89 (10.11)
	P-value	-	< 0.001	< 0.001	< 0.001	0.002	< 0.001
Education	Illiterate	143 (36.3)	44.56 (11.04)	29.8 (13.21)	54.34 (8.57)	55.06 (12.25)	45.94 (8.73)
	Primary school	149 (37.8)	51.99 (12.93)	40.25 (16.08)	57.46 (10.13)	59.79 (14.31)	52.37 (10.87)
	Secondary school and higher	102 (25.9)	59.61 (11.92)	47.55 (16.52)	65.83 (9.7)	68.77 (14.15)	60.44 (10.68)
	P-value	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Income	Low (< 1000)	224 (56.8)	48.43 (12.82)	35.44 (17.47)	56.22 (9.98)	57.9 (14.73)	49.50 (11.39)
	acceptable	170 (43.2)	54.23 (13.27)	41.38 (15.41)	60.86 (10.49)	63 (13.9)	54.86 (11.06)
	P-value	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Disease duration (yr)	≥ 3 yr	51 (12.9)	56.12 (13.81)	41.88 (16.86)	61.7 (12.31)	65.48 (14.81)	56.29 (12.18)
	4-7	93 (23.6)	51.09 (10.95)	37.3 (16.15)	57.52 (9.29)	59.47 (12.48)	51.34 (10.06)
	≤ 7 yr	250 (63.5)	50.33 (13.89)	38 (16.91)	58.19 (10.43)	59.7 (15.03)	51.55 (11.78)
	P-value	-	0.019	0.26	0.058	0.029	< 0.001
	< 25	72 (20.2)	55.21 (12.90)	42.08 (17.32)	60.77 (12.09)	64.36 (15.85)	55.6 (12.53)
	25-29.9	148 (41.6)	54.2 (13.01)	39.25 (15.68)	59.2 (10.26)	62.15 (14.22)	53.7 (11.66)
	≥ 30	136 (38.2)	47.97 (12.85)	35.27 (17.46)	57.16 (9.98)	58.23 (13.79)	49.66 (11.08)
	P-value	-	< 0.001	0.014	0.052	0.008	0.001
HbA1c	< 7	180 (47.2)	51.11 (12.70)	38.02 (16.02)	57.8 (10.84)	60.1 (14.12)	51.76 (11.07)
	≥ 7	201 (52.8)	51.21 (14.01)	37.96 (17.35)	58.89 (10.25)	60.83 (14.75)	52.22 (12.05)
	P-value	-	0.938	0.969	0.136	0.62	0.696
	NORMAL	195 (49.6)	53.83 (10.78)	38.33 (15.11)	60.4 (9.57)	64.73 (11.81)	54.32 (9.74)
Kessler psychological distress	MILD	72 (18.3)	52.01 (13.65)	41.97 (18.03)	57.47 (10.25)	60.84 (12.61)	53.07 (11.57)
	MODERATE	52 (2.13)	50.96 (14.41)	39.19 (19.07)	58.57 (12.12)	59.76 (16.61)	52.12 (13.91)
	SEVER	74 (18.8)	43.9 (15.76)	34.14 (17.23)	54.33 (66.10)	48.87 (15.22)	45.31 (11.66)
	P-value	-	< 0.001	0.042	< 0.001	< 0.001	< 0.001
Functional limitation	No	106 (26.9)	61.25 (10.89)	47.47 (15.85)	63.05 (11.16)	67.91 (14.37)	59.92 (10.79)
	Moderate	78 (19.8)	54.92 (11.51)	44.34 (18.16)	61.12 (9.62)	64.79 (12.13)	56.29 (10.18)
	Sever	210 (53.3)	44.8 (11.35)	31.44 (13.25)	55.17 (9.26)	54.91 (13.17)	46.58 (9.23)
	P-value	-	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Treatment	Oral medication	223 (57.4)	53.57 (12.59)	38.69 (15.71)	58.97 (10.37)	61.69 (14.64)	53.11 (11.09)
	Oral medication + insulin injection	164 (42.2)	48.92 (13.64)	37.82 (17.96)	58.07 (10.55)	58.78 (14.38)	50.9 (11.97)
	P-value	-	0.007	0.883	0.161	0.101	0.12
Comorbidities	No	102 (25.9)	58.93 (11.79)	42.23 (18.29)	63.37 (10.57)	66.51 (14.82)	57.76 (11.75)
	1	207 (52.5)	50.82 (12.07)	38.28 (16.42)	57.56 (10.03)	60.49 (13.19)	51.79 (10.66)
	≥ 2	85 (21.6)	43.05 (12.99)	33.74 (14.41)	54.83 (9.39)	52.75 (13.88)	46.09 (10.09)
	P-value	-	< 0.001	0.002	< 0.001	< 0.001	< 0.001

HRQOL: Health related quality of life.

Table 4 Multivariate linear regression models of significant factors predicting health related quality of life domains among diabetic people referring to diabetes clinics of Tabriz, 2015

HRQOL domains	Variables	B (SE)	Beta	*P*-value	95%CI of B		Adjusted R2
					Lower	Upper	
Physical health	Education	3.35 (0.83)	0.198	< 0.001	1.77	4.93	0.436
	BMI	-1.55 (0.75)	-0.087	0.039	-3.12	0.07	
	Functional limitation	-4.79 (0.77)	-0.229	< 0.001	-6.11	-3.07	
	Kessler psychological distress	-1.98 (0.46)	-0.174	< 0.001	-2.90	-1.06	
	Comorbidies	-4.05 (0.85)	-0.210	< 0.001	-5.73	-2.37	
Social relationship	Age	-4.65 (1.2)	-0.212	< 0.001	-7.01	-2.28	0.279
	Education	5.3 (1.15)	0.246	< 0.001	3.03	7.56	
	Functional limitation	-4.05 (1.11)	-0.208	< 0.001	-6.24	-1.87	
Psychological health	Education	3.52 (0.94)	0.190	< 0.001	1.67	5.38	0.353
	Functional limitation	-3.94 (0.9)	-0.234	< 0.001	-5.72	-2.15	
	Comorbidies	-3.72 (1.0)	-0.176	< 0.001	-5.69	-1.75	
	Kessler psychological distress	-3.96 (0.55)	-0.317	< 0.001	-5.04	-2.88	
Environment	Education	4.3 (0.73)	0.318	< 0.001	2.86	5.75	0.257
	Comorbidies	-2.37 (0.78)	-0.154	0.003	-3.91	-0.83	
	Kessler psychological distress	-1.33 (0.43)	-0.135	0.004	-2.07	-0.38	
	Functional limitation	-1.77 (0.7)	-0.145	0.012	-3.17	-0.38	
	Income	2.13 (1.02)	0.101	0.037	0.12	4.14	
Total HRQOL score	Education	4.12 (0.7)	0.278	< 0.001	2.73	5.5	0.433
	Functional limitation	-3.59 (0.67)	-0.267	< 0.001	-4.92	-2.26	
	Age	1.48 (0.73)	-0.098	0.044	-2.93	-0.03	
	Kessler psychological distress	-2.02 (0.41)	-0.203	< 0.001	2.83	-1.21	
	Income	1.98 (0.97)	0.085	0.044	0.05	3.9	
	Comorbidities	-2.41 (0.75)	-0.143	0.001	-3.89	-9.41	

HRQOL: Health related quality of life.

psychological distress and level of income) (Table 4).

DISCUSSION

HRQOL is one of the most important assessment indices of health cares in chronic disease[24]. In this study, HRQOL based on the WHOQOL-BRIFE and its correlates among people with type 2 diabetes was examined. Based on these findings, the mean of overall HRQOL was 52.11 ± 11.53 which was similar to other studies that have also shown that HRQOL dimensions of diabetes patients was moderate[25-27], while some studies reported the lower score of the mean of overall HRQOL[28-30]. Based on these findings, in all dimensions, men had higher average HRQOL than women (55.46 ± 11.34 and 50.39 ± 11.27 in males and females, respectively), which was consistent with the result obtained in studies conducted by Rasouli *et al*[31], Khalde *et al*[32] and Redekop *et al*[33]. These studies attributed women's low HRQOL score to biological and psychological differences (women's menopause and sensitivity in dealing with the disease). But Saadatjoo *et al*[34] reported that women's HRQOL score obtained in different dimensions was higher than men, which is different from the results obtained in the present research. Some studies also have shown no significant association between gender and HRQOL[35]. In the present study, the lowest and highest HRQOL scores were obtained in mental and social dimensions, respectively. The score was different in other studies due to socioeconomic status and cultural conditions as well as collection tools. The findings of the present study showed a significant association between the HRQOL of patients, and factors including age, income, BMI, level of education, functional limitation, psychological distress, and number of comorbidities which was consistent with the study conducted by Didarloo *et al*[36]. There was a significant relationship between BMI and HRQOL so that by increasing BMI levels, HRQOL level was decreased. The results of regression analysis showed that there was a relationship between BMI and HRQOL in terms of physical dimension (b = -1.5), which were consistent with many studies conducted in this area[30,37,38]. The association between age and HRQOL was consistent with many studies so that the lowest and highest mean HRQOL scores were obtained in young and elderly patients, respectively[19,39,40]. The results of the present study showed that there was a significant relationship between level of education and all HRQOL dimensions so that people with higher education levels also had better QOL, which is consistent with findings obtained in different studies[12,41,42]. Moreover, the findings of the present study indicated that the frequency of comorbidities in patients was associated with a reduced HRQOL and this relationship was significant in the physical, psychological and environmental dimensions based on the results obtained in multiple regression analysis[3,43]. There was a negative correlation between functional limitation and HRQOL among people with type 2 diabetic in the current study. This means that increasing functional limitation score was indicative of the fact that patients faced limitation in doing their daily

activities, which in turn reduced their HRQOL. There were no similar studies for comparison purposes in this context.

The results of the current study showed that the psychological distress had negative effects on the average HRQOL of patients and led to reduced HRQOL in these people. The results of multiple regression analysis were indicative of a significant relationship between psychological distress and all HRQOL dimensions (except social dimension). These findings are consistent with other studies done in this area[24,44]. In the present study, there was a reverse relationship between duration of diabetes, and HRQOL scores; but after adjustment for other variables it was no longer significant in any of HRQOL dimensions. Studies[45,46] also indicated that there was no significant relationship between duration of diabetes and HRQOL, which confirmed the results of the present study.

In conclusion, the findings of the present study showed that age, level of education, income, BMI, functional limitation, psychological distress and number of comorbidities have a decisive role on HRQOL of patients with type2 diabetes. So, it is important to improve the HRQOL by considering above predictors as an appropriate mechanism for public health interventions for type 2 diabetes. Therefore, correcting lifestyle and increasing facilities of clinics providing service can be an effective step to improve the QOL of patients.

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COMMENTS

Background

One of the important issues in the care of chronic diseases such as diabetes is to investigate the quality of their life, which significantly affects one's physical-psychological performance and social communication. Although, some studies showed that demographic factors, socio-economic status, presence of comorbid conditions, and diabetes control affect health related quality of life (HRQOL) among people with type 2 diabetes, a comprehensive assessment of a range of epidemiologic and clinical factors related to the quality of life (QOL) among people with type 2 diabetes in this area is needed.

Research frontiers

Diabetes an emerging health problem in Iran and will continue to rise in the next decades. Considering that East Azerbaijan province, is among provinces, in which diabetes is highly prevalent and the different climatic, socio-cultural conditions, lifestyle of the area as well as the low quality of diabetes care can affect the QOL, a comprehensive assessment of clinical and epidemiological correlates of QOL can provide a more clear picture of the problem in order to implement an appropriate public health interventions.

Innovations and breakthroughs

To the knowledge, limited studies in this area have been conducted to assess QOL and a range of different epidemiological and clinical factors specially there is no information about the association between functional limitation, and psychological distress and QOL in Iran. This study designed to capture a more details about the QOL and its correlates using a valid questionnaires and trained interviewers.

Applications

QOL is considered as an outcome measure therefore identification of any modifiable factor associated with that could be of interest for further intervention. Diabetes will continue to rise; health policy makers need to be updated about the required information in order to implement the new interventional programs and also to enhance the current practice related to diabetes care.

Terminology

QOL: Individuals' perception of their position in life in terms of culture, value system where they live, goals, expectations, standards and priorities; HRQOL: A subjective issue that is measured using different dimensions include physical, mental and social functions; Kessler psychological distress (K10): A 10-item questionnaire intended to measure the level of distress based on questions about anxiety and depressive symptoms over the recent 4 wk.

Peer-review

The paper is interesting and has been developed with appropriate methodology.

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Randomized Clinical Trial

Impact on dietary intake of a self-directed, gender-tailored diabetes prevention program in men

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Abstract

AIM

To investigate changes in dietary intake following a 6-mo

randomised controlled trial of the self-directed, gender-tailored type 2 diabetes mellitus (T2DM) Prevention Using LifeStyle Education (PULSE) program in men.

METHODS

Men aged 18-65 years, with a body mass index (BMI) 25-40 kg/m^2, and at high risk for developing T2DM were recruited from the Hunter Region of New South Wales, Australia. Eligible participants were randomised into one of two groups: (1) waitlist control; or (2) PULSE intervention. Dietary intake was assessed at baseline and immediately post-program using the Australian Eating Survey food frequency questionnaire and diet quality measured using the Australian Recommended Food Score (ARFS).

RESULTS

One hundred and one participants (n = 48, control; n = 53, intervention, mean age 52.3 ± 9.7 years, BMI of 32.6 ± 3.3 kg/m^2) commenced the study. Following the active phase, differences between groups were observed for proportion of total energy consumed from healthful (core) foods (+7.6%EI, P < 0.001), energy-dense, nutrient-poor foods (-7.6%EI, P < 0.001), sodium (-369 mg, P = 0.047), and diet quality (ARFS) (+4.3, P = 0.004), including sub-scales for fruit (+1.1, P = 0.03), meat (+0.9, P = 0.004) and non-meat protein (+0.5, P = 0.03).

CONCLUSION

The PULSE prevention program's nutrition messages led to significant improvements in dietary intake in men at risk of T2DM.

Key words: Dietary intake; Diet quality; Men; Diabetes prevention program; Self-directed

Core tip: In the context of type 2 diabetes mellitus (T2DM) prevention programs, only recently has the effect on diet quality been reported. However, no studies have examined the effect on diet of a program designed exclusively for men. This study reports on the dietary outcomes following the self-directed T2DM Prevention Using LifeStyle Education (PULSE) program. Following completion of the PULSE program, men receiving the intervention significantly reduced intake of energy-dense, nutrient-poor foods and portion size. In addition, the intervention group increased overall diet quality and greater variety within healthful food groups.

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INTRODUCTION

Despite concerted public health efforts, the prevalence of diabetes continues to increase worldwide. Between 2013 to 2015, the worldwide prevalence of diabetes increased by 33 million to an estimated 415 million adults[1,2], with the prevalence expected to increase to 642 million in the next 25 years[1]. The economic burden on national health systems attributed to diabetes is significant, with USD 612 billion spent worldwide or 11% of total health expenditure[3]. In higher income countries, approximately 87%-91% of those with diabetes have type 2 diabetes mellitus (T2DM)[1]. Further, global estimates place 318 million adults at risk of developing the condition, with an additional 163 million individuals estimated to have impaired glucose tolerance by 2040[1].

Lifestyle risk factors such as a high body mass index (BMI), sub-optimal diet quality and lack of physical activity are key targets underpinning T2DM prevention programs, such as the Diabetes Prevention Program in the United States[4] and the Diabetes Prevention Study in Finland[5]. In the years following these seminal studies, many diabetes prevention programs have been evaluated including successful adaptations for various populations and different settings[6-8].

These T2DM prevention programs promote moderate weight loss by improving physical activity and dietary behaviours. Programs containing a combination of diet and physical activity components have demonstrated efficacy with regard to weight loss and improvements in glucose regulation[9,10]. Specific dietary changes following the diabetes prevention programs have included a reduction in total energy intake and favourable shifts in macronutrient composition (*e.g.*, reduction in total and saturated fat intake)[9]. Only recently have changes in diet quality or amounts of foods (as opposed to energy or nutrients) been reported both immediately following completion of the intervention[11,12], as well as over the long-term for periods of 5-10 years later[13,14]. To date, no studies have reported the effect on diet of a diabetes prevention program tailored for men. Therefore, this study examines changes in dietary intake, in particular diet quality, among Australian men following the 6-mo randomised controlled trial of the self-directed, gender-tailored Prevention Using LifeStyle Education (PULSE) program.

MATERIALS AND METHODS

The PULSE randomised controlled trial evaluated the efficacy of a self-directed, diabetes prevention program tailored for men at risk of T2DM. The study was conducted in Newcastle, Australia in 2012-2013 after receiving ethical approval (H-2012-0232) from the University of Newcastle Human Research Ethics Committee and registration with

the Australian New Zealand Clinical Trials Registry (ACTRN 12612000721808).

Detailed information regarding the rationale, study design and methods are reported elsewhere[15,16]. Briefly, emerging evidence supports the use of gender-tailored approaches, in particular for weight loss[17,18]. Our previous research has found gender-tailored programs to be effective in producing weight loss in men[19-21]. However, T2DM prevention programs predominantly involve both men and women with results reported collectively[9]. Therefore, the PULSE program aimed to address this gap in the evidence through the evaluation of a T2DM prevention program designed exclusively for men.

Males aged 18-65 years, with a BMI 25-40 kg/m^2 and at high risk for developing T2DM (with a score $\geqslant$ 12 as self-reported *via* the Australian Diabetes risk assessment tool[22]) were recruited. Men with pre-existing diabetes or other serious medical conditions, recent significant weight loss (> 5% in past 6 mo), currently participating in other weight loss programs, and without access to a mobile phone were ineligible. Study recruitment used advertisements across various modes including print and online, workplace emails and research participant registers. Participants were stratified by age and BMI, and randomised to either the intervention group or waitlist control. The active intervention period was 6 mo with the waitlist control group receiving the PULSE program at the completion of active phase.

As previously reported, the program was effective in reducing clinical risk factors of T2DM[16]. In summary, a significant mean difference favouring the intervention group over control was observed for change in body weight (-5.5 kg and -5.3%, both $P < 0.001$), BMI (-1.8 kg/m^2, $P < 0.001$), and waist circumference (umbilicus -5.4 cm, $P < 0.001$; narrowest point -6.2 cm; $P < 0.001$). In addition, significant changes were found for measures of glucose regulation in the intervention group with improvements in HbA1c (-0.2%, $P = 0.002$), fasting insulin (-3.0 mIU/L, $P = 0.002$) and measures of insulin resistance (HOMA-IR; -0.4, $P = 0.002$) and insulin sensitivity (QUICKI; +0.02, $P = 0.006$).

The PULSE program

The current paper reports the secondary dietary outcome data, and therefore the methods focus on the intervention components used to target changes in diet and eating behaviours. Underpinned by Social Cognitive Theory, the PULSE program aimed to promote modest weight loss through changes in key dietary and physical activity behaviours. The program addressed key theoretical constructs relating to goal setting and planning, positive outcome expectations, seeking social support, promoting behavioural self-monitoring, and increasing self-efficacy, as described in detail previously[15,16]. Following randomisation, participants in the intervention group were provided with information and equipment resource packs and briefly orientated to the contents by a research team member[15,16].

The PULSE handbook contained dietary information and focused on four main messages: (1) key nutrients and their role in the body; (2) dietary composition, focusing on amount and quality of carbohydrate [*i.e.*, lower glycaemic index (GI)], fat, protein and fibre, and using the plate model to represent appropriate meal portion sizes; (3) variety within core (healthful) foods, particularly vegetables; and (4) suggestions for the composition (*e.g.*, low-moderate GI) of breakfast, lunch and dinner. In addition, participants were provided with the Self-Help, Exercise and Diet using Information Technology (SHED-IT) program[19,23]. This handbook provided information on general weight loss principles including setting daily energy intake targets, goal setting for eating and activity behaviours, self-monitoring tools for tracking weight, waist circumference and step counts. In addition, participants were provided with a calorie counter[24] and instructions for the accompanying CalorieKing website (www.calorieking.com.au). Participants were encouraged to self-monitor dietary intake and physical activity (both for at least 4 d per week) and weight (once per week).

Outcome measures

Assessments occurred at baseline and following the program (6 mo). Usual dietary intake was assessed using the validated, semi-quantitative food frequency questionnaire, the Australian Eating Survey (AES)[25] at baseline and following the program completion. The AES comprises 120 food items with 15 supplementary questions on food behaviours. Standard adult portion sizes for each food item were derived from National Nutrition Survey data or from the product standard serving size (*e.g.*, slice of bread)[26]. Participants were asked to recall the frequency of food consumption over the past 3 mo, with individual responses for each food or food type. Frequency options ranged from "Never" up to "$\geqslant$ 4 times/d", but varied depending on the food, with some drinks items up to "$\geqslant$ 7 glasses/d". Nutrient intakes were computed from the most current food composition database of Australian foods, the Australian AusNut 1999 database (All Foods) Revision 17, primarily and AusFoods (Brands) Revision 5.

The validated Australian Recommended Food Score (ARFS)[27] assesses diet quality and variety within the food groups relative to the Australian Guide to Healthy Eating within the Australian Dietary Guidelines (ADGs)[28]. The ARFS uses a sub-set of 70 AES food items and comprises eight sub-scales from a range of healthful or core food groups (*e.g.*, vegetables, fruit, grains, meats, non-meat proteins, dairy) with total score ranging from 0 to 73. For most items AES frequency response options are collapsed into two categories "once per week or more" or "less than once per week or never". A higher total score is indicative

Table 1 Baseline dietary intake characteristics (*n* = 101)

	Control (*n* = 48)	Intervention (*n* = 53)	All participants (*n* = 101)
Dietary intake			
EI (kJ/d)	11761 ± 3550	11014 ± 3143	11369 ± 3346
Core foods (%EI)	56.9 ± 9.3	55.8 ± 12.1	56.3 ± 10.8
Non-core foods (%EI)	43.1 ± 9.3	44.2 ± 12.1	43.7 ± 10.8
Protein (%EI)	17.5 ± 2.4	17.3 ± 2.8	17.4 ± 2.6
Carbohydrate (%EI)	45.6 ± 7.1	44.8 ± 5.1	45.1 ± 6.1
Fat (%EI)	30.6 ± 4.6	30.5 ± 5.0	30.6 ± 4.8
Saturated fat (%EI)	12.7 ± 2.2	12.7 ± 2.6	12.7 ± 2.4
Monounsaturated fat (%EI)	11.4 ± 2.1	11.3 ± 2.1	11.4 ± 2.0
Polyunsaturated fat (%EI)	3.8 ± 1.0	3.8 ± 0.9	3.8 ± 0.9
Alcohol (%EI)	6.9 ± 5.9	7.8 ± 7.2	7.4 ± 6.6
Fibre (g/d)	31.5 ± 12.0	29.1 ± 8.9	30.2 ± 10.5
Sodium (mg/d)	2834.3 ± 975.3	2662.1 ± 865.2	2743.9 ± 918.6
ARFS (maximum score)			
Total ARFS (73)	32.2 ± 10.9	30.3 ± 7.8	31.2 ± 9.4
Vegetables (21)	12.1 ± 5.0	11.1 ± 4.2	11.6 ± 4.6
Fruit (12)	4.5 ± 3.2	3.8 ± 2.5	4.1 ± 2.9
Meats (7)	3.1 ± 1.5	2.6 ± 1.3	2.9 ± 1.4
Non-meat protein (6)	2.0 ± 1.1	1.8 ± 1.0	1.9 ± 1.1
Grains (13)	5.1 ± 2.1	5.0 ± 1.7	5.1 ± 1.9
Dairy (11)	4.2 ± 2.0	4.4 ± 1.6	4.3 ± 1.8
Sauces (2)	0.9 ± 0.7	1.1 ± 0.8	1.0 ± 0.7
Water (1)	0.3 ± 0.5	0.4 ± 0.5	0.3 ± 0.5
Portion size			
Potato[1]	1.7 ± 0.5	1.6 ± 0.6	1.7 ± 0.5
Vegetables[3]	1.1 ± 0.6	1.1 ± 0.6	1.1 ± 0.6
Casserole[3]	2.0 ± 0.5	1.9 ± 0.5	1.9 ± 0.5
Steak[2]	1.8 ± 0.6	1.8 ± 0.5	1.8 ± 0.6

Data is presented as mean ± SD. Portion size coded as per Hodge *et al*[31] as follows: Never eat = 0, less than image A = 0.4, equal to image A = 0.5, between images A and B = 0.75, equal to image B = 1.0, between images B and C = 1.5, equal to image C = 2.0, greater than image C = 2.5. [1]*n* = 100 participants; [2]*n* = 97 participants; [3]*n* = 99 participants. EI: Energy intake; ARFS: Australian Recommended Food Score.

of more optimal nutrient intakes[27,29], greater variety within the core food groups and alignment with ADGs.

Portion size for four common foods (potato, vegetables, steak and casserole) was assessed separately using food photographs from the Dietary Questionnaire for Epidemiological Studies, version 2[30,31]. Three photographs are displayed, representing the 25th, 50th (median), and 75th percentiles of portion sizes for adult men and women[31] to indicate the portion size typically consumed, with eight response options ranging from "not eating the food at all" up to "more than the amount represented".

Statistical analysis

Statistical analysis was conducted using Stata version 13. Between group differences in completers (those who did complete all 6-mo follow-up measures) *vs* non-completers (those who did not complete all 6-mo follow-up measures, including those lost to follow-up) were assessed using *t* tests for continuous data and χ^2 tests for categorical data.

Changes in dietary intake over the 6-mo intervention were analysed using linear mixed models according to the intention-to-treat principle. Outcomes were assessed for the impact of treatment (intervention compared to control), time [baseline and immediately post program (*i.e.*, 6 mo)] and group by time interaction. Models were adjusted for participant age and socioeconomic status (SES), which were specified a priori. The coefficient and *P*-value from the mixed model testing the difference between groups in change from baseline to 6 mo was used to determine the effect of the intervention on each outcome (significance level, $P < 0.05$). The statistical methods of this study were reviewed by Daniel Barker from the University of Newcastle.

RESULTS

A total of 101 participants were recruited to the study. Participant baseline dietary intakes are reported in Table 1. Nineteen participants (*n* = 6 control; *n* = 13 intervention) were lost to follow-up (not able to attend the assessment sessions or were unable to be contacted). An additional two participants in the intervention group attended the follow-up session, but did not complete the AES at 6-mo, leaving a total of 21 non-completers and 80 completers from the original 101 participants whom commenced the study. At baseline, non-completers reported lower intakes of total energy, protein and carbohydrates compared to completers. The mean age of the participants was 52.3 ± 9.7 years (range 20-66 years) and the most frequently reported highest education level was a certificate or trade qualification (60%). The mean BMI of the sample was 32.6 ± 3.3 kg/m^2, and ranged from 25.7-41.0 kg/m^2.

Changes in dietary intakes of participants are reported in Table 2. At follow-up, significant mean differences between groups favouring the intervention group were identified for %EI from healthful (core) foods (+7.6%EI; $P < 0.001$), energy-dense, nutrient-poor foods (-7.6%EI, $P < 0.001$), protein (+1.3%EI, $P = 0.03$), and polyunsaturated fat (-0.4%EI, $P = 0.02$), as well as sodium intake (-369 mg, $P = 0.047$). Between group differences were observed in ARFS diet quality, with the intervention group achieving a greater improvement in mean total score (+4.3, $P = 0.004$) and subscales of fruit (+1.1, $P = 0.03$), meat (+0.9, $P = 0.004$) and non-meat protein (+0.5, $P = 0.03$). Greater reductions in portion sizes were achieved in the intervention group for potato (-0.9, $P = 0.002$), steak (-0.9, $P = 0.002$) and casserole (-0.7, $P = 0.01$) compared to controls, however there was no change in vegetable portion size.

DISCUSSION

The PULSE self-directed T2DM prevention program for men resulted in significant improvements in usual

Table 2 Change in dietary intake by group at 6 mo (*n* = 101)

	Change baseline to 6 mo, mean (95%CI)		
	Control (*n* = 48)	Intervention (*n* = 53)	Diff between groups
Dietary intake			
EI (kJ/d)	-315.5 (-1412.9, 781.9)	-1618.1 (-2568.4, -667.9)[b]	-1298.5 (-2737.7, 140.6)
Core foods (% EI)	2.0 (-0.4, 4.5)	9.6 (7.0, 12.3)[d]	7.6 (4.0, 11.2)[d]
Non-core foods (%EI)	-2.0 (-4.5, 0.4)	-9.6 (-12.3, -7.0)[d]	-7.6 (-11.2, -4.0)[d]
Protein (%EI)	0.5 (-0.4, 1.4)	1.9 (1.1, 2.6)[d]	1.3 (0.1, 2.5)[a]
Carbohydrate (%EI)	-0.8 (-2.5, 0.8)	0.1.1 (-2.5, 0.4)	-0.4 (-2.6, 1.9)
Fat (%EI)	-0.5 (-1.9, 1.0)	0.06 (-1.5, 1.4)	0.4 (-1.6, 2.5)
Saturated fat (%EI)	0.004 (-0.7, 0.8)	-0.6 (-1.4, 0.08)	-0.6 (-1.7, -0.4)
Monounsaturated fat (%EI)	-0.2 (-0.8, 0.4)	0.09 (-0.5, 0.7)	0.3 (-1.0, 0.5)
Polyunsaturated fat (%EI)	-0.3 (-0.6, -0.04)[a]	0.1 (-0.1, 0.4)	0.4 (0.1, 0.8)[a]
Alcohol (%EI)	0.7 (-0.5, 1.9)	-0.9 (-2.5, 0.7)	-1.6 (-3.6, 0.4)
Fibre (g/d)	-0.2 (-3.3, 3.0)	-0.1 (-2.6, 2.4)	-0.01 (-4.0, 4.0)
Sodium (mg/d)	-151.3 (-433.2, 130.5)	-519.8 (-753.0, -286.6)[d]	-368.5 (-732.0, -4.9)[a]
ARFS (maximum score)			
Total ARFS (73)	-0.2 (-2.2, 1.7)	4.1 (2.0, 6.3)[d]	4.3 (0.1.4, 7.2)[b]
Vegetables (21)	0.5 (-0.6, 1.5)	2.0 (0.7, 3.3)[b]	1.5 (-0.1, 3.2)
Fruit (12)	-0.08 (-0.8, 0.6)	1.0 (0.3, 1.7)[b]	1.1 (-0.1, 2.1)[a]
Meats (7)	0.3 (-0.7, 0.03)	0.5 (0.1, 1.0)[a]	0.9 (0.3, 1.4)[b]
Non-meat protein (6)	0.07 (-0.3, 0.2)	0.4 (0.05, 0.8)[a]	0.5 (-0.03, 0.9)[a]
Grains (13)	0.07 (-0.5, 0.3)	0.3 (-0.3, 0.8)	0.3 (-0.4, 1.0)
Dairy (11)	-0.2 (-0.6, 0.2)	-0.2 (-0.7, 0.2)	-0.06 (-0.7, 0.5)
Extras (2)	-0.01 (-0.2, 0.2)	-0.06 (-0.3, 0.2)	-0.05 (-0.4, 0.3)
Water (1)	0.04 (-0.07, 0.2)	0.1 (-0.01, 0.3)[a]	0.1 (-0.08, 0.3)
Portion size			
Potato	-0.03 (-0.4, 0.3)	-0.9 (-1.3, -0.4)[d]	-0.9 (-1.4, -0.3)[b]
Vegetable	-0.1 (-0.5, 0.3)	-0.06 (-0.5, 0.3)	-0.04 (-0.5, 0.6)
Steak	-0.2 (-0.5, 0.2)	-1.1 (-1.6, -0.6)[d]	-0.9 (-1.5, -0.3)[b]
Casserole	-0.2 (-0.5, 0.2)	-0.9 (-1.3, -0.5)[d]	-0.7(-1.3, -0.2)[a]

Significant differences within and between groups: [a]$P < 0.05$; [b]$P < 0.01$; [d]$P < 0.001$. Portion size coded as per Hodge *et al*[31] as follows: Never eat = 0, less than image A = 0.4, equal to image A = 0.5, between images A and B = 0.75, equal to image B = 1.0, between images B and C = 1.5, equal to image C = 2.0, greater than image C = 2.5. EI: Energy intake; ARFS: Australian Recommended Food Score.

dietary intake, including a reduction in intakes of energy-dense, nutrient-poor foods and increased overall diet quality and variety within healthful food groups and fruit, non-meat protein and meat ARFS subscales. Of note portion sizes for potatoes, steak and casserole were significantly reduced in the intervention group *vs* the control group. Though not significant, changes in the desired direction were observed for increased vegetable variety and decreased alcohol intake (%EI).

For men in the PULSE program intervention group, the increased diet quality was accompanied by an increased percentage of total energy from healthful foods and a %EI from energy-dense, nutrient-poor foods. Despite a reduction in mean total energy intake of approximately 1300 kJ/d ($P = 0.08$), dietary macronutrient composition remained relatively stable, with a small but significant increase of +1.3% in %EI from protein in the intervention group. This is in contrast to the United States Diabetes Prevention Program which reported a similar reduction in total energy intake among those in the lifestyle intervention group, however total fat intake decreased by 6.6% of total energy intake following the first 6 mo[4]. Similar reductions in total energy were also observed in the Finnish Diabetes Prevention Study following the intensive phase at 1 year[32]. These findings, combined with the increases in diet quality, suggest individuals in the PULSE intervention program replaced energy-dense, nutrient-poor foods with healthful food choices.

Most studies reporting on dietary outcomes immediately following major diabetes prevention program interventions have only reported on changes in total energy and/or nutrient intakes[9]. Few studies have evaluated changes in diet quality as assessed using an a priori defined diet quality index or score, or intakes of individual food groups. Miller *et al*[12] reported significant within group improvements in overall diet quality scores (+4.6, $P < 0.01$) measured using the Alternative Healthy Eating Index following a 4-mo group-based diabetes prevention program. In another study, Block *et al*[11] reported changes in food habits associated with higher diet quality, such as significant changes in the consumption frequency of fruits and vegetables (increases) and sweets and refined carbohydrate foods (decreases) found among those with prediabetes who received a 6-mo automated web-based diabetes prevention program.

Findings from interventions aimed at the prevention of T2DM, including the current PULSE trial, are consistent with meta-analyses[33,34] which support a significant association between higher overall diet quality, as assessed by a score or index, and a decreased risk of T2DM. Specifically in men with a high BMI, diets of higher quality have been associated with a reduction in the incidence of T2DM[35]. Findings from the current study also add to the emerging evidence for a lower risk of developing T2DM in those with a higher diet quality[36,37]. Greater dietary diversity within core food groups has also been associated with a lower risk of metabolic syndrome[38], and to be a predictor of weight loss and reduced waist circumference within a weight loss intervention[39]. In particular, diets that are diverse in the variety of fruits and vegetables consumed are associated with a lower risk of T2DM[37]. Recent meta-analyses on the amounts of fruits and vegetables consumed in relation to T2DM risk confirm the positive relationship between higher intakes of fruits and vegetables and lower risk of developing diabetes[40,41], including a dose-response relationship with a 6% and 13% lower risk for each 1 serve increase in fruit intake and each additional 0.2 serve of green-leafy vegetables, respectively[40].

The changes in diet in those receiving the PULSE intervention program are encouraging, especially given the self-directed nature of the program and compare

favourably to traditional intensive T2DM prevention programs delivered using one-on-one or group or combination approaches. Given the increasing prevalence of T2DM and associated risk factors, such as excess body weight, poor diet and physical inactivity, evidence of the effectiveness of low intensity programs are gaining momentum[7]. However, modifications to the traditional diabetes prevention programs are considered necessary for translation to non-research or "real-world" settings[42] and to promote sustainability of effective programs through delivery of these programs at scale. Delivery of programs using various media and technologies, such as DVD or web- or mobile-based platforms, have demonstrated effectiveness in terms of weight loss[6,11,43], while other diabetes prevention programs have begun to provide higher intensity interventions through the supplementation of mobile applications with remote support from a trained health coach[44,45].

The dietary changes observed by men in the PULSE intervention group support the observed improvements in weight status, waist circumference and measures of glucose regulation[16]. These findings demonstrate that participants adhered to the dietary messages contained in the PULSE program, in particular increasing variety within healthful food groups and reducing portion sizes. This indicates that a gender-tailored, self-directed program can result in desirable dietary changes that reduce T2DM risk. Despite, the benefits demonstrated by the PULSE program immediately following completion, the long-term impact on the maintenance of healthy eating behaviours remain to be established.

Although a validated food frequency questionnaire was used to measure dietary intake, the use of a self-administered measure is subject to inherent errors of self-reported dietary data[46] and is a limitation of the current study. The effect of measurement error in the context of intervention studies is an emerging area of research and recommendations to use a biomarker of dietary intake to calibrate the primary measure of intake[47] were outside the scope of the PULSE pilot study. While the dietary outcome findings should be interpreted in light of this, the use of objective measures for the anthropometric and biochemical outcomes is a strength of the current study, and improvements in these variables are likely to reflect the positive dietary changes observed for the intervention group.

In addition to reductions in anthropometric measures and improved glucose regulation, the self-directed, gender-tailored PULSE program resulted in significant improvements in dietary intake. This included a reduction in intake of energy-dense, nutrient-poor foods and portion size, increased overall diet quality and greater variety of healthful foods, especially within fruit, meat, and non-meat protein food groups.

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COMMENTS

Background

Dietary outcomes following diabetes prevention programs have primarily focused on reporting changes in energy and macronutrient intakes; effects on diet quality have only begun to be investigated. Also, most past programs have included both men and women, were not gender-tailored, and had little or no separate reporting of effects on men and women. This study investigated effects on dietary intakes and diet quality of the self-directed, gender-tailored Prevention Using LifeStyle Education (PULSE) program for type 2 diabetes mellitus (T2DM) prevention in men.

Research frontiers

Emerging evidence supported the use of gender-tailored programs for men in the context of weight loss, however no programs developed for T2DM prevention had been specifically tailored for men. The PULSE program aimed to address this gap.

Innovations and breakthroughs

The PULSE program's nutrition messages led to significant improvements in dietary intake and diet quality, as well as decreasing clinical risk factors for T2DM, in men at risk of T2DM, suggesting that gender-tailoring in this group may be important for achieving healthful dietary behaviour changes in men.

Applications

These findings offer support for the use of gender-tailored dietary messages in the context of dietary advice for the prevention of T2DM, in particular in relation to increasing variety within healthful food groups.

Terminology

Diet quality assessment provides an indication of adherence to dietary guidelines and healthy eating patterns. Diet quality can be assessed *via* indices or scores that evaluate types of foods consumed and variety within food groups, and in some instances, intakes of selected nutrients.

Peer-review

The study is very interesting from a clinical practice point of view.

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ORIGINAL ARTICLE

Basic Study

Expression of *matrix metalloproteinase-11* is increased under conditions of insulin resistance

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Abstract

AIM

To investigate *matrix metalloproteinase-11* (*MMP-11*) expression in adipose tissue dysfunction, using *in vitro* and *in vivo* models of insulin resistance.

METHODS

Culture of mouse 3T3-L1 preadipocytes were induced to differentiation into mature 3T3-L1 adipocytes. Cellular insulin resistance was induced by treating differentiated cultured adipocytes with hypoxia and/or tumor necrosis factor (TNF)-α, and transcriptional changes were analyzed in each condition thereafter. For the *in vivo* studies, *MMP-11* expression levels were measured in white adipose tissue (WAT) from C57BL/6J mice that underwent low fat diet or high-fat feeding in order to induce obesity and obesity-related insulin resistance. Statistical analysis was carried out with GraphPad Prism Software.

RESULTS

MMP-11 mRNA expression levels were significantly higher in insulin resistant 3T3-L1 adipocytes compared to control cells (1.46 ± 0.49 *vs* 0.83 ± 0.21, respectively;

P < 0.00036). The increase in *MMP-11* expression was observed even in the presence of TNF-α alone (3.79 ± 1.11 *vs* 1 ± 0.17, *P* < 0.01) or hypoxia alone (1.79 ± 0.7 *vs* 0.88 ± 0.1, *P* < 0.00023). The results obtained in *in vitro* experiments were confirmed in the *in vivo* model of insulin resistance. In particular, *MMP-11* mRNA was upregulated in WAT from obese mice compared to lean mice (5.5 ± 2.8 *vs* 1.1 ± 0.7, respectively; *P* < 3.72E-08). The increase in *MMP-11* levels in obese mice was accompanied by the increase in typical markers of fibrosis, such as collagen type Ⅵ alpha 3 (*Col6α3*), and fibroblast-specific protein 1.

CONCLUSION

Our results indicate that dysregulation of *MMP-11* expression is an early process in the adipose tissue dysfunction, which leads to obesity and obesity-related insulin resistance.

Key words: Metalloproteinase-11; Insulin resistance; Type 2 diabetes; Fibrosis; Hypoxia; Tumor necrosis factor-α; Inflammation

Core tip: 3T3-L1 mature adipocytes are widely used as a cellular model of obesity. We treated 3T3-L1 adipocytes with tumor necrosis factor-α and/or hypoxia for 24 h to induce insulin resistance. *Matrix metalloproteinase-11* (*MMP-11*) expression levels were upregulated in insulin resistant adipocytes, as compared to untreated control cells. This observation was confirmed *in vivo*, in white adipose tissue from insulin-resistant obese mice. Therefore, our results suggest that *MMP-11* could play a role in the dysfunction of adipose tissue, which leads to insulin resistance and type 2 diabetes. Further work is necessary to understand better the functional role of *MMP-11* in this context.

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INTRODUCTION

Insulin resistance is a pathological condition in which insulin target tissues fail to properly respond to insulin. It is more frequently associated with overweight and obesity, and constitutes a prominent feature of type 2 diabetes (T2D) and the metabolic syndrome[1,2]. In the past decades, research findings have substantially improved our understanding of the pathophysiology of insulin resistance, thanks to the identification of new genetic defects and molecular events that underlie the abnormalities that occur in both peripheral insulin action and insulin secretion[3-7]. Particular interest in this field has been devoted to the investigation of obesity, as it is considered the major risk factor for insulin resistance, which leads to the development of T2D and other obesity-associated insulin resistant states. Therefore, because of the parallel increasing prevalence of obesity and metabolic diseases, much research has been recently focused on the role of adipose tissue, previously considered as a fat storage tissue only. Evidence from the last years has established the involvement of adipose tissue in the production of hormones and numerous other biologically active molecules collectively called "adipokines" that are implicated in metabolic and inflammatory pathways[8]. Based on the new view of adipose tissue as an endocrine organ, new insights have been gained over the last years into the mechanisms linking adipose tissue to insulin resistance, although the entire sequelae of events that initially trigger adipose tissue dysfunction still remain poorly defined.

The *matrix metalloproteinase-11* (*MMP-11*; also known as stromelysin-3) is a proteinase *enzyme* that belongs to the family of metalloproteinases, and is involved in remodeling and degradation of extracellular matrix (ECM). Unlike other MMPs that are secreted in an inactive form to be then activated extracellularly, *MMP-11* is maturated in the Golgi's apparatus and secreted in an active form[9]. *MMP-11* is implicated in tissue remodeling during embryogenesis, tissue involution and metamorphosis, and in biological process of tissue repair after trauma[10]. In addition, as shown in *in vivo* studies, *MMP-11* plays a role in tumor development and progression. In particular, cancer cells, by inducing the adjacent fat cells to express *MMP-11*, may contribute to modify the ECM, thereby favoring cancer cell migration into the connective tissue, during the initial step of the invasive process[11]. In this regard, the involvement of *MMP-11* in certain types of cancers (*i.e.,* breast, colorectal and lung) has been confirmed in clinical studies, in which it has also been established that higher expression of *MMP-11* correlates with tumor aggressiveness and lower survival rate among affected patients[12]. However, although the numerous studies carried out up to date, both *in vitro* and *in vivo*, the precise molecular target(s) of *MMP-11* and their specific role in normal and pathological conditions have not yet been clarified. It has been demonstrated that active *MMP-11* is primarily responsible for the digestion of collagen Ⅳ and Ⅵ, fibronectin, alpha 2-macroglobulin and insulin-like grow factor binding protein 1 (IGFBP1)[13,14]. However, all these substrates are not specific for this enzyme as they can be also cleaved by other MMPs.

In the present study, we investigated the expression of *MMP-11* in an *in vitro* model of insulin resistance, and in a murine diet-induced model of obesity.

MATERIALS AND METHODS

Cell culture

3T3-L1 mouse preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL

streptomycin and maintained at 37 ℃ in 5% CO_2 humidified atmosphere. As soon as the confluence was reached, cells were induced to differentiate as reported previously[15,16]. In brief, the differentiation process was started through the addition of 500 μmol/L of 3-isobutyl-1-methylxanthine (IBMX), 1 μmol/L of dexamethasone and 1 μg/mL of insulin. The cells were incubated for three days in the differentiation medium, followed by 2 d of treatment with DMEM containing 1 μg/mL insulin. The medium was replaced every two days and experiments were performed using day 8 to day 12 mature adipocytes.

Induction of insulin resistance in vitro

To induce insulin resistance, mature 3T3-L1 adipocytes were treated with 2.5 nmol/L tumor necrosis factor (TNF)-α, and simultaneously incubated in hypoxic conditions for 24 h[17]. Before inducing insulin resistance, 3T3-L1 adipocytes were cultured in DMEM at low glucose concentration (1 g/L) and 0.5% BSA, plus rh-TNF-α, and put in the hypoxic chamber (1% O_2, 5% CO_2) at 37 ℃ for 24 h. Control cells were incubated in the same conditions, but in normal atmosphere (21% O_2).

Total RNA isolation and reverse transcription

Total RNA was extracted from white adipose tissue (WAT) and 3T3-L1 cells, using Trizol reagent (Invitrogen), according to the manufacturer's instructions[18]. RNA concentration was measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, United States), and its quality confirmed on agarose gel. One microgram of RNA sample was used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), in the presence of the following reagents: 10 × RT Buffer, 100 mmol/L dNTP mix, 10 × RT Random Primers and 0.50 U/μL Multiscribe Reverse Transcriptase. The cDNA thermal-profile was 25 ℃ for 10 min, 37 ℃ for 120 min and the enzyme was inactivated at 85 ℃ for 5 min.

Quantitative PCR

Relative quantification was performed to measure *MMP-11* expression, using a real-time thermocycler (Eppendorf Mastercycler ep realplex ES). One microliter of cDNA and 0.2 μmol/L of each primers were mixed with SYBR Green RealMasterMix (Eppendorf). S9 and 18S were used as internal reference controls. Primers were designed for mouse *MMP-11*, S9 and 18S, using the Primer3web version 4.0[19,20], according to sequences from the GeneBank database. Amplification conditions were: 2 min at 95 ℃ and three step-cycle of 95 ℃ for 15 s, 58 ℃ for 20 s and 68 ℃ for 20 s, for a total of 40 cycles.

Western blot

Cells were lysed as described previously[21]. Cellular protein (20 μg) was resolved on 10% SDS-PAGE, transferred to PVDF membrane (Immobilon-PSQ 0.2 μm Millipore ISEQ00010), blotted for 2 h with blocking solution (5% non-fat dry milk), then incubated overnight at 4 ℃ with primary antibody against *MMP-11* (Santa Cruz sc8836 dilution 1:1000), followed by incubation for 1 h at room temperature with a secondary antibody linked to horseradish peroxidase. Immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham).

Animals

Five week-old male C57BL/6J mice were housed in individual cages and maintained on 12 h light-dark cycle with controlled temperature (25 ℃) and humidity (50% ± 5%), and with free access to water. To induce obesity, ten mice were fed ad libitum with HFD containing 60% calories from fat, 20% from carbohydrates, and 20% from protein for 15 wk time period. Six additional mice (control group) were fed for the same time (15 wk) with low fat diet (LFD) containing 10% calories from fat, 70% from carboydrates, and 20% from protein. Intraperitoneal insulin tolerance test (IITT) was performed following previously described procedures[5,22], using human insulin (Human Actrapid, Novo Nordisk), 0.25 U/kg body weight, then measuring blood glucose levels at 0, 15, 30, 45, 60 min after insulin injection. At the end of 15 wk, mice were euthanized by cervical dislocation, epididimal WAT tissue rapidly removed and frozen in liquid nitrogen *until* analysis.

All animal procedures were performed according to the guidelines of the Charité universitätsmedizin Berlin and were approved by the Landsamt für Gesundheit und Soziales (Berlin, Germany) for the use of laboratory animals and according to the current version of the German Law on protection of animals for scientific purposes.

Statistical analysis

All calculations were analyzed with GraphPad Prism Software. Mean values were compared with *t*-test. A P value < 0.05 (two tailed) was considered significant.

RESULTS

MMP-11 expression in 3T3-L1 cells

We first examined the expression of *MMP-11* during 3T3-L1 adipogenesis. Total RNA was prepared at different stage of adipocyte cell differentiation and *MMP-11* mRNA expression levels were measured. As shown in Figure 1, *MMP-11* mRNA abundance was low in 3T3-L1 pre-adipocytes, increased in confluent culture cells, reaching maximum expression in mature 3T3-L1 adipocytes (Figure 1).

MMP-11 expression in in vitro insulin resistance

To induce insulin resistance *in vitro*, fully differentiated 3T3-L1 adipocytes were treated with TNF-α (2.5 nmol/L) and at the same time incubated in hypoxia (1% O_2) for 24 h. Then, *MMP-11* mRNA and protein expression levels were measured. As shown in Figure 2A, a clear increase in both mRNA and protein expression of the *MMP-11* proteinase was observed in insulin resistant 3T3-L1 cells, as compared to normal, non-insulin

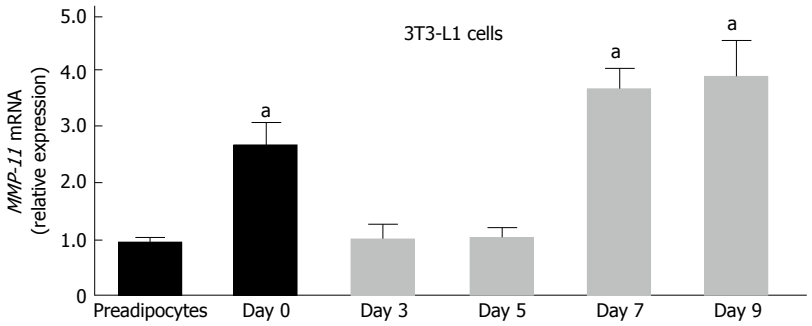

Figure 1 Expression of *matrix metalloproteinase-11* during adipocyte differentiation in 3T3-L1 cells. Total RNA was extracted from 3T3-L1 cells at preadipocyte and confluent (day 0) stages, and after induction of differentiation (days 3, 5, 7 and 9). *MMP-11* mRNA expression was measured by RT-PCR. Results are the means ± SE of three independent experiments, each performed in triplicate. [a]*P* < 0.05 *vs* undifferentiated preadipocytes. *MMP-11*: *Matrix metalloproteinase-11*.

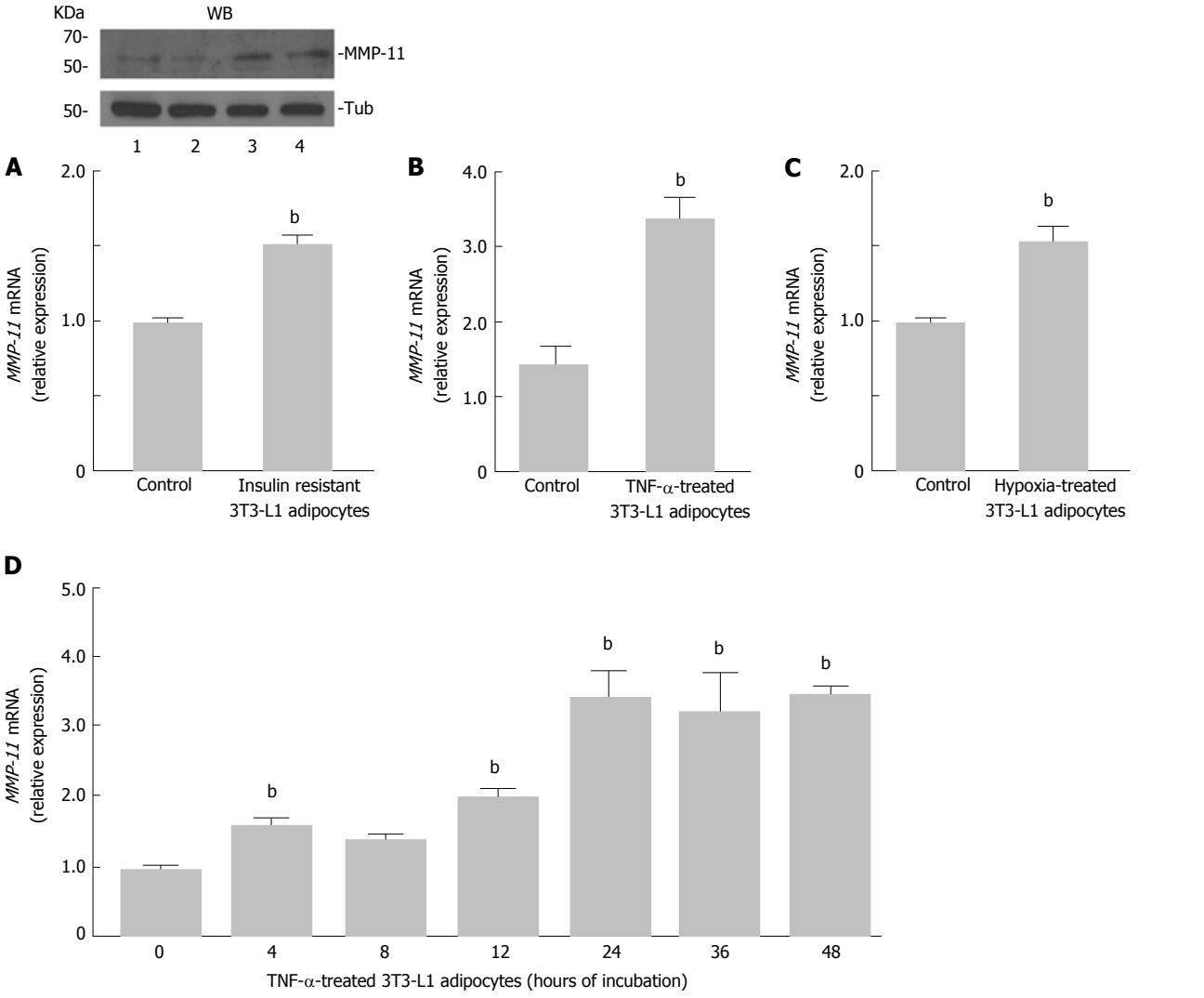

Figure 2 *Matrix metalloproteinase-11* expression in insulin-resistant 3T3-L1 adipocytes. A: Fully differentiated 3T3-L1 adipocytes were co-treated with TNF-α (2.5 nmol/L) and hpoxia (O_2 1%) for 24 h, and *MMP-11* mRNA was measure by RT-PCR. Results are the means ± SE of three independent experiments, each in triplicate. [b]*P* < 0.01 *vs* untreated (control) cells. A representative western blot (WB) of *MMP-11* is shown for each experimental condition. Lanes: 1 and 2, *MMP-11* protein expression in untreated 3T3-L1 cells (control); 3 and 4, *MMP-11* protein expression in insulin-resistant 3T3-L1 cells. Tubulin (Tub), control of protein loading; B: 3T3-L1 adipocytes were treated with TNF-α alone, at a final concentration of 2.5 nmol/L, and *MMP-11* mRNA levels were measured 24 h later by RT-PCR. Results are the means ± SE from three independent experiments. [b]*P* < 0.01 *vs* untreated control cells; C: 3T3-L1 adipocytes were incubated in normoxic (control) or hypoxic condition (O_2 1%) for 24 h, total RNA was extracted and the expression of *MMP-11* was determined by RT-PCR. Results are the means ± SE from three independent experiments in triplicate. [b]*P* < 0.01 *vs* control; D: Time-course of *MMP-11* mRNA expression in differentiated 3T3-L1 adipocytes, in the presence of TNF-α (2.5 nmol/L) alone. *MMP-11* mRNA was measured by RT-PCR at the indicated time points, after TNF-α treatment. Results are the means ± SE from three independent experiments, each in triplicate. [b]*P* < 0.01 *vs* time 0. *MMP-11*: *Matrix metalloproteinase-11*.

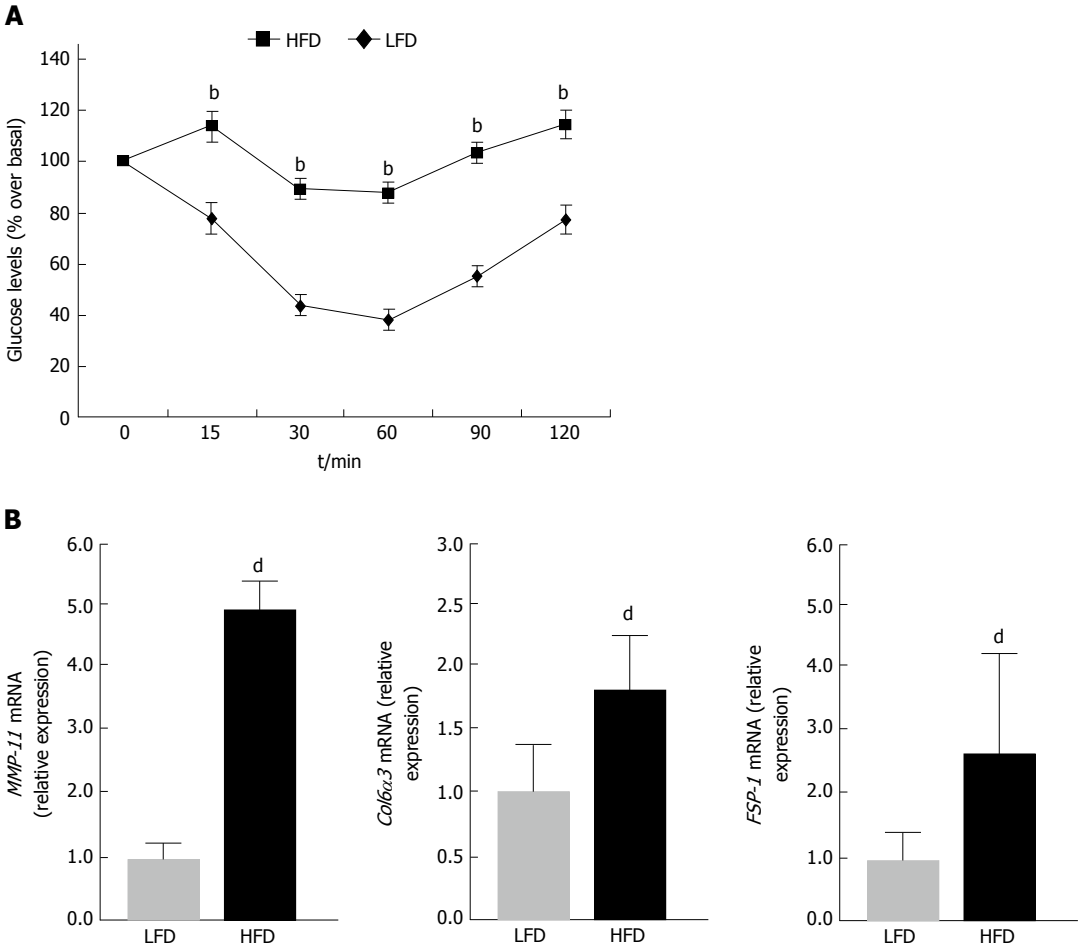

Figure 3 Intraperitoneal insulin tolerance test and the expression of *matrix metalloproteinase-11*, collagen type Ⅵ alpha 3 and fibroblast-specific protein 1 in mice under different dietary conditions. A: IITT. Insulin tolerance was assessed in 12 h fasted mice, intraperitoneally injected with insulin (0.25 U/kg body weight). LFD (n = 6); HFD (n = 10). $^{b}P < 0.01$ *vs* LFD; B: MMP-11, *Col6α3* and *FSP-1* mRNA expression in WAT of mice fed a low-fat (LFD) or high-fat (HFD) diet (n = 6 per each group). Results are the means ± SE of three independent measurements from each animal. $^{d}P < 0.001$ *vs* LFD, for each group. MMP-11: *Matrix metalloproteinase-11;* IITT: Intraperitoneal insulin tolerance test; *Col6α3*: Collagen type Ⅵ alpha 3; *FSP-1*: Fibroblast-specific protein 1.

resistant 3T3-L1 adipocytes. To better understand the effect of each treatment on *MMP-11* expression, we carried out separate experiments in which *MMP-11* mRNA levels were measured in fully differentiated 3T3-L1 adipocytes treated with either 2.5 nmol/L of TNF-α for 24 h, or subjected to 24 h hypoxia alone. As shown in Figure 2B, *MMP-11* mRNA abundance was approximately four-fold higher in TNF-α treated cells compared to untreated 3T3-L1 adipocytes, thereby indicating that upregulation of *MMP-11* expression can be at least in part regulated by the pro-inflammatory TNF-α molecule. On the other hand, hypoxia alone induced a slight but significant increase of *MMP-11* mRNA expression compared to normoxia (Figure 2C). A time-course study of *MMP-11* mRNA expression, using TNF-α alone over a 48 h period, showed that *MMP-11* mRNA levels were significantly increased already after 4 h and this increase was maintained thereafter, reaching a plateau level at 24 h of exposure (Figure 2D).

MMP-11 expression in in vivo insulin resistance

In attempt to validate the results obtained *in vitro*, in insulin-resistant *3T3-L1* adipocytes, mRNA expression studies were carried out also *in vivo*, in a mouse model of insulin resistance[23,24]. To this end, ten male mice were fed with HFD for 15 wk, whereas six other mice, which were used as controls, were subjected to normal chow diet, for the same time period. At the end of the diet treatment, mice fed with HFD were obese relative to control mice (43.6 ± 2.1 g *vs* 27.5 ± 1.7 g, respectively; P < 170829E-06), and developed insulin resistance as assessed by IITT (Figure 3A). Gene expression analysis to evaluate the levels of *MMP-11* was then performed in both groups of mice. As shown in Figure 3B, *MMP-11* mRNA was significantly higher in WAT from diet-induced obese mice than in WAT from lean mice, indicating that hyperexpression of *MMP-11* may also occur *in vivo*, in the whole animal, after induction of an insulin-resistant state, thereby suggesting that abnormal activation of *MMP-11* may have direct consequences on the molecular mechanism(s) related to adipocyte dysfunction. In this regard, the expression profile of two major fibrosis marker genes, collagen type Ⅵ alpha 3 (*Col6α3*) and fibroblast-specific protein 1 (*FSP-1*), was also measured in parallel experiments. As shown in Figure 3C, both these markers were significantly upregulated in WAT from obese mice compared to lean animals (Figure 3C), highlighting the possibility, in our

obese mouse model, for an ECM dysregulation that would support the hypothesis that this ECM remodeling could indeed exert an adverse effect on adipocyte functions.

DISCUSSION

Adipose tissue is surrounded by ECM elements that provide the right support for adipocyte cell growth and expansion, and maintenance of adipocyte specific functions. Alterations in the organization and flexibility of the ECM as a cause of adipose tissue dysfunction have been reported[25], together with the observation that several MMPs could be involved in these adverse events[26].

In the present work, we focused our attention on the *MMP-11* and its activation in conditions of insulin resistance, either *in vitro*, in 3T3-L1 mouse adipocytes, or *in vivo*, in obese mice. For the first time, in the present study, we show that *MMP-11* was upregulated both in insulin resistant cells treated with TNF-α and/or hypoxia (two elements frequently associated with obesity), and in adipose tissues from insulin-resistant obese mice, suggesting that a direct link may exist between activation of *MMP-11* and adipocyte cell dysfunction. Our data are consistent with previous observations that adipokines and hypoxia can alter the expression of MMPs. In this regard, it has been shown that TNF-α upregulated MMP-9 expression in the osteoblast-like MC3T3-E1 cell line[27], while in another study it was found that MMP-2 expression increased in response to hypoxia[28]. Furthermore, an involvement of both MMP-2 and *MMP-11* in ECM degradation and collagen accumulation, associated with adipocyte dysfunction, was reported previously[29]. It can be hypothesized that upregulation of *MMP-11* in insulin resistance may reflect the increase of nuclear proinflammatory transcription factor(s) whose effective role needs to be investigated.

Fibrosis is considered a new hallmark of the pathological dysfunction of WAT[25]. In our study, it is also interesting to note the alteration in the expression of genes related to fibrosis (*Col6α3* and *FSP-1*) in WAT from nutritionally induced obese mice. A link between *Col6α3* and *MMP-11* has been reported before[29]. Thus, our data in this context well support previous reports that overexpression of MMPs, *via* degradation of ECM, could be implicated in adipose tissue remodeling[25], and this can play a role in the pathological dysfunction of adipose tissue, which leads to insulin resistance.

Our results appear to challenge findings obtained by studying the *MMP-11* knock-in transgenic mouse[30], in which protection from diet-induced obesity was reported, together with a condition of enhanced glucose tolerance and insulin sensitivity due to increased IGF-I bioactivity[30]. The explanation for these divergent results may reside in the fact that overexpression of active *MMP-11* in the skin of the transgenic animal may not necessarily reflect the situation *in vitro*, in 3T3-L1 adipocytes and *in vivo*, in WAT from diet-induced obese mice. On the other hand, the existence of compensatory mechanisms/changes that may contribute to counteract genetic manipulation has been proposed[31-35].

Overall, although further studies are still necessary to clarify the role of *MMP-11* in insulin resistance, we believe our findings may contribute to shed light on the early process of adipose tissue dysfunction commonly associated with obesity and obesity-related insulin resistance.

COMMENTS

Background

Insulin resistance is a common metabolic disorder, in which peripheral target tissues fail to respond adequately to insulin, thereby predisposing to type 2 diabetes and other dysmetabolic conditions. More recent discoveries have now strengthened the hypothesis that adipose tissue dysfunction could be the primum movens in the development of insulin resistance. Therefore, studies have been focused on exploring the molecular mechanism(s) underlying adipocyte dysfunction.

Research frontiers

Matrix metalloproteinases (MMPs) are a class of endopeptidases that contribute to the degradation of the extracellular matrix components. It has been discovered that they are involved in adipogenesis and remodelling of adipose tissue. A better understanding of the role and function of MMPs in adipose tissue will open new frontiers of investigations.

Innovations and breakthroughs

For the first time, the authors demonstrate that overexpression of *MMP-11* occurs in *in vitro* and *in vivo* models of insulin resistance.

Applications

This study suggests that *MMP-11* could be involved in the early stage of obesity-related insulin resistance. Thus, as a secreted serum protein, *MMP-11* could serve as an early biomarker of adipose tissue dysfunction. Research in this area will lead to advancement in understanding the pathophysiology of insulin resistance, as well as advancement in drug development and therapy.

Peer-review

The paper is straight forward, well written, and it adds novel information on the topic.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical profile of diabetes at diagnosis among children and adolescents at an endocrine clinic in Ghana

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Author contributions: Ameyaw E contributed to study design, conducted the study, and contributed to the manuscript; Asafo-Agyei SB contributed to concept and assisted in conduct of the study; Thavapalan S analysed the data, prepared the figures, and reviewed the manuscript; Middlehurst AC assisted with study design and review of data; Ogle GD designed the study, interpreted the results, and was the lead writer on the manuscript.

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Abstract

AIM

To determine the clinical features of diabetes in children and adolescents in Ghana.

METHODS

Retrospective review of clinical features of all children and adolescents with new-onset diabetes seen at the paediatric endocrinology clinic of Komfo Anokye Teaching Hospital in Kumasi, from February 2012 to Auguest 2016.

RESULTS

One hundred and six subjects presented with diabetes. Ninety (84.9%) were diagnosed by clinical features and family history as type 1, and 16 (15.1%) type 2. For type 1 subjects, age range at diagnosis was 0.9-19.9 year (y), peak age of onset 12-13 year, and 3.3% were < 5 year, 21.1% 5- < 10 year, 45.6% 10- < 15 year and 30.0% 15- < 20 year. Seventy-one point one percent were female. Common clinical features were polyuria (100%), polydipsia (98.9%), and weight loss (82.2%). Mean BMI SD was -0.54, range -3.84 to 2.47. 60.0% presented in diabetic ketoacidosis (DKA). Nine had infections at onset (skin, abscess, leg ulcer). Mean

± SD HbA1c at diagnosis was 12.7% ± 1.9% (115 ± 21 mmol/mol). Four have since died: Hypoglycaemia (2), recurrent DKA (1), osteosarcoma (1). Two other type 1 cases died of DKA at presentation in emergency before being seen by the paediatric endocrinologist. Crude mortality rate including these 2 cases was 32.2/1000 patient years. Type 2 cases were 81% female, age of onset 9-19 year. Mean BMI SD was 1.49, range -0.87 to 2.61. Forty-three point eight percent presented in DKA. All type 2 cases had acanthosis nigricans. Overall, 9.8% did not have home refrigeration, most using clay pot evaporative cooling for insulin storage.

CONCLUSION

Type 1 occurs with a female preponderance and high DKA rates. Type 2 also occurs. Typology based on clinical features is difficult. Community and professional awareness is warranted.

Key words: Children; Diabetes; Developing countries; Ghana; Mortality

Core tip: In this study of 106 consecutive new diagnoses of diabetes in young people < 20 years in a tertiary referral centre in Ghana, type 1 predominated (85%) with the remaining cases clinically diagnosed as type 2. Both types had a female preponderance. Type 1 peak age of onset was 12-13 years. All type 2 subjects had acanthosis nigricans. Most presented in ketoacidosis signifying a lack of awareness of presentation features. Clinic numbers quickly rose due to availability of supplies and expertise. Further typology studies are indicated to further define diabetes type.

Ameyaw E, Asafo-Agyei SB, Thavapalan S, Middlehurst AC, Ogle GD. Clinical profile of diabetes at diagnosis among children and adolescents at an endocrine clinic in Ghana. *World J Diabetes* 2017; 8(9): 429-435 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i9/429.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i9.429

INTRODUCTION

Understanding the presentation and types of diabetes in children and youth in any particular country is essential in improving awareness and care. Ghana is a less-resourced country in West Africa. There is no published data on clinical features of young Ghanaians with diabetes and, as with many low-income countries[1], there is little public health sector support and also lack of awareness amongst both health workers and the general society[2]. Insulin is only intermittently available from the government health service, and blood glucose meters and strips and HbA1c testing are not provided by the Ghana National Health Insurance Scheme. The families must often buy these supplies, often at premium prices[3], which many cannot afford to do[2].

The lack of awareness leads to misdiagnosis and mismanagement. Ketoacidosis is very common at initial presentation in Africa[2,4-6], and can mimic infections and acute medical conditions[7-10].

This study determined the clinical features of children and adolescents presenting with diabetes at the Paediatric Endocrine Clinic, Komfo Anokye Teaching Hospital (KATH) at Kumasi, a tertiary referral centre for northern Ghana. This clinic has been supported since 2012 by the International Diabetes Federation (IDF) Life for a Child Program[11] with provision of insulin, blood glucose meters and strips, insulin syringes, HbA1c testing, education materials, and mentoring.

MATERIALS AND METHODS

Study subjects

A total of 106 subjects were enrolled, all < 20 years of age at diagnosis. They included all subjects being followed at the Paediatric Endocrine Clinic on 24/02/2012 as well as all new diagnoses until 31/08/2016. During this period, two other subjects < 20 years old (both female, aged 12 and 15 years old respectively) presented with diabetic ketoacidosis (DKA) and died in the emergency department. They were not seen by the pediatric endocrinologist or in the clinic, and no further information is available. Therefore, they were included in the mortality rate calculation, but excluded from the remainder of the analysis. The study was approved by the institutional ethics board and subjects gave informed consent.

Demographic data

Date of birth and sex was recorded, as well as date of diagnosis.

Clinical parameters

Diabetes was diagnosed according to standard World Health Organization criteria[12]. Determination of the type of diabetes was made by the local investigators according to available clinical features and history. Type 1 patients generally had lower body mass index (BMI), more rapid symptom onset, and were more sensitive to insulin. Type 2 patients had higher BMI, acanthosis nigricans, and needed more insulin with time, with insulin requirements falling sharply in those started on metformin. The presence of polyuria, polydipsia, weight loss, malnutrition and ketoacidosis at the time of diagnosis were recorded. Body weight and height were measured by electronic scales and stadiometer respectively with subjects wearing light-weight clothing and without shoes. BMI was then calculated. BMI SD scores were calculated using World Health Organization standards[13,14].

Ketoacidosis was defined by clinical features along

with an elevated blood glucose and ketonuria (blood gas measurements are generally not available). Family history of type 1 diabetes, and history of other medical conditions were also recorded.

Biochemical parameters

Blood glucose was measured in a laboratory *via* venous sample. HbA1c was measured using a Clover analyzer (Infopia, Anyang, South Korea).

Socioeconomic parameters

The following information was collected for each subject: Whether the mother or father was living with the subject, mother's and father's educational level, who was the primary caregiver, whether the primary caregiver was literate, time spent travelling to clinic, and average weekly household income. It was also recorded whether the subject was at school, whether diabetes was limiting school attendance, and whether they were in the appropriate grade for age, and how well overall the young person was psychologically coping with their diabetes (rated as poor, average or good). Finally, the method of insulin storage was recorded.

Crude mortality rate was calculated as the total number of deaths divided by the sum of the periods from the commencement of the study, or from the date of diagnosis if they were diagnosed after the study commenced. It is expressed as mortality per 1000 patient years.

Statistical analysis

Data and descriptive statistics were managed in Excel. Unpaired *t*-test and χ^2 tests were done using the Social Science Statistics on-line calculators[14]. Significance was set as < 0.05.

RESULTS

One hundred and six subjects with diabetes were seen at the paediatric endocrine clinic. Ninety (84.9%) were diagnosed by clinical features and family history as type 1, and 16 (15.1%) type 2.

Type 1 subjects

Table 1 shows age of onset and gender of the 90 type 1 subjects, as well as BMI, BMI SD score, presence of DKA at diagnosis, and blood glucose and HbA1c at diagnosis. Figure 1A shows the distribution of age of onset. Three point three percent were < 5 years, 21.1% 5- < 10 years, 45.6% 10- < 15 years and 30.0% 15- < 20 years. Common clinical features at diagnosis were polyuria (100.0%), polydipsia (98.9%), and weight loss (82.2%). Nine (10%) had infections at onset (tinea capitis, abscess, leg ulcer, vaginal candidiasis).

Nine type 1 subjects had a first-degree relative with type 1: Sister (two subjects), brother (three), sister and brother (two), two brothers (one), mother (one), with one other subject having a grandmother with type 1. The number of insulin injections each day was two for 17 (18.9%) subjects, three for 24 (26.7%), five for 47 (52.2%) and unknown for two (2.2%). The type of insulin was pre-mixed for 11 (12.2%) subjects, and short-acting combined with long-acting for 79 (87.8%).

Four of the 106 patients have since died: One from metastatic osteosarcoma (diagnosed well after onset of type 1), two from hypoglycemia at home (2 years after diagnosis), and one from a recurrent episode of DKA (2 years after diagnosis). Two others died in emergency department during treatment of DKA at diagnosis, and were not seen by the paediatric endocrinologist (see Methods). Crude mortality rate for the type 1 patients was six deaths per 186 patient years (*i.e.*, 32.2 deaths per 1000 patient years).

Type 2 subjects

For the 16 type 2 cases, Table 1 shows age of onset and gender, as well as BMI, BMI SD score, presence of DKA at diagnosis and blood glucose and HbA1c at diagnosis. Figure 1B shows age of onset. Six point three percent were 5- < 10 years, 68.7% 10- < 15 years and 25.0% 15- < 20 years. Common clinical features at diagnosis were polyuria (100.0%), polydipsia (100.0%), and weight loss (93.8%). All type 2 subjects had acanthosis nigricans. None had infections at onset. One had substantial visual loss at diagnosis, of uncertain aetiology. Three subjects had first degree relatives with type 2, and two others had a second-degree relative. Four subjects (25.0%) were treated with metformin only, six (37.5%) with insulin only, five (31.3%) with metformin together with insulin and one (6.3%) also with glibenclamide. No subject with type 2 died.

Increase in clinic numbers

Figure 2 shows the rapid increase in clinic numbers in the 4 years from June 2012 to June 2016 - clinic numbers were censused at the end of every half-year.

Socioeconomic factors

The mother was living with the subject in 83 (78.3%) cases and the father in 78 (73.6%). The mother's educational level was primary school in 31 (29.2%) cases, high school in 26 (24.5%), tertiary in 10 (9.4%), no schooling in 38 (35.8%) and unknown in 1 (0.9%). The father's educational level was primary school in 26 (24.5%) cases, high school in 31 (29.2%), tertiary in 23 (21.7%), no schooling in 21 (19.8%) and unknown in 5 (4.7%). The primary caregiver was the mother in 75 (70.8%) cases, father in 15 (14.2%), sister in 3 (2.8%), brother in 2 (1.9%), grandmother in 4 (3.8%), aunt in 6 (5.7%) and self in 1 (0.9%). The primary caregiver was literate in 79 (74.5%) cases. Twenty-four (22.6%) families had to travel long distances (> 2 h travelling time each way) for supplies and review. The average weekly household income was 63 USD and the range was 5-625 USD. Ninety-six (90.6%) subjects were attending school. Diabetes was limiting attendance at

Table 1 Characteristics of type 1 and type 2 subjects at diagnosis

	Type 1	Type 2	Difference
Number (%)	90 (84.9)	16 (15.1)	$P < 0.001$
Male: Female ratio	1:2.5	1:4.3	Not significant
Age at diagnosis (range), yr	0.9-19.9	9.0-18.7	-
Age at diagnosis (mean ± SD), yr	12.6 ± 3.8	13.6 ± 2.3	Not significant
Peak age at diagnosis, yr	12-13	13-14	-
Diabetic ketoacidosis at onset (%)	54 (60.0)	7 (43.8)	Not significant
BMI at onset (mean; range)	18.1; 12.5-34.7	27.8; 17.6-38.2	-
BMI SD score at onset (mean; range)	-0.54, -3.84-2.47	1.49, -0.87-2.61	$P < 0.001$
HbA1c at diagnosis (mean ± SD) (%) (mmol/mol)	12.7 ± 1.9 (115 ± 21)	12.8 ± 1.5 (116 ± 16)	Not significant

BMI: Body mass index; HbA1c: Glycosylated haemoglobin.

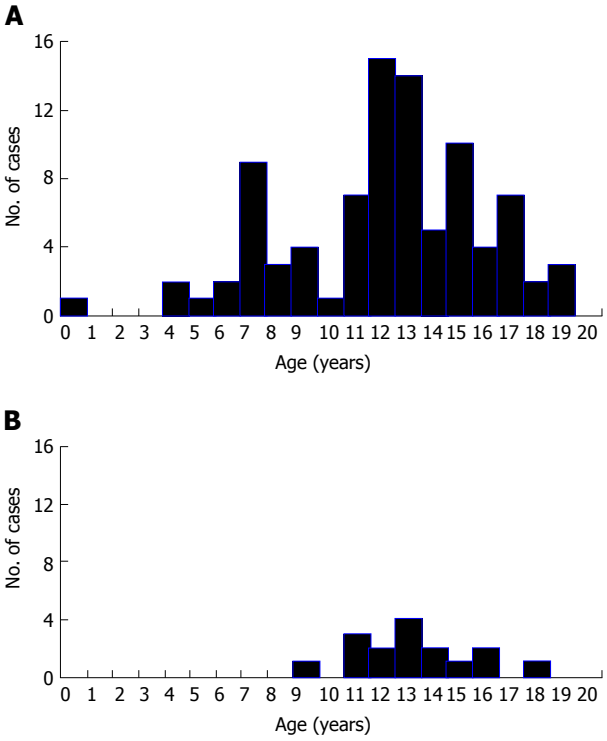

Figure 1 Age at diagnosis of subjects < 20 years of age with diabetes in Kumasi, Ghana. A: Type 1 diabetes: Age at diagnosis; B: Type 2 diabetes: Age at diagnosis.

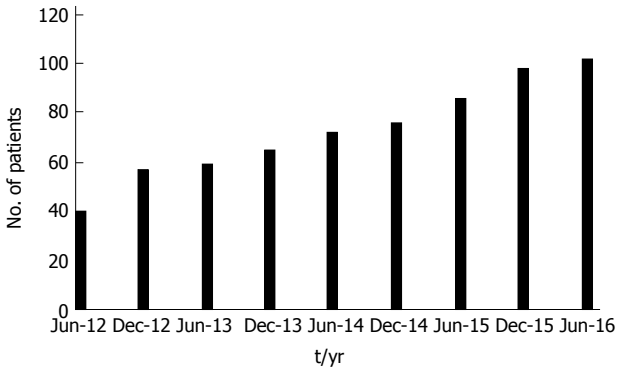

Figure 2 Numbers of patients with diabetes being seen at the paediatric endocrine clinic in Kumasi, Ghana.

school for 44 (45.8%) subjects, not limiting attendance for 51 (53.1%) and unknown for 1 (1.0%). In addition, 18 (18.8%) were not in the appropriate grade for their age, 76 (79.2%) were in the appropriate grade, and 2 (2.1%) unknown. Diabetes coping abilities were assessed as poor for 12 (11.3%) subjects, average for 37 (34.9%), good for 55 (51.9%) and unknown for 2 (1.9%). Ninety-five (89.6%) subjects were literate or learning at school, 8 (7.5%) were not literate and 3 (2.8%) unknown. Insulin storage method was a refrigerator at the family home for 92 subjects (90.2%), for two a refrigerator outside the home (2.0%) and for eight clay pot evaporative cooling (7.8%).

DISCUSSION

There are very limited published data on diabetes in young people in Ghana. The International Diabetes Federation Diabetes Atlas estimates an incidence of type 1 diabetes of 2.9 per 100000 children < 15 years per annum and a prevalence of 18.0 per 100000 children < 15 years: An estimated 1800 children in the country[15,16]. This is however based on a small study in Nigeria in 1992[17]. It is possible that the current Ghanaian incidence is different from this estimate, and the prevalence/incidence ratio is likely to be substantially lower as the Atlas estimates do not assume any mortality[16]. In Ghana, it is likely that many children and young adults with diabetes die before they are diagnosed, or die during the first episode of DKA or early in ongoing management. DKA is frequently misdiagnosed at first as another condition - with a legion of alternatives including pneumonia, gastroenteritis, malaria, typhoid, appendicitis and a number of other conditions[1,7-10]. At a training workshop organised by Ghana Society of Pediatric Endocrinology and Diabetes (GSPED) in August 2016, some participants from district and regional hospitals admitted that most of their patients with DKA die. Indeed, two centres admitted that all such patients have died during management. The rate of DKA at onset in type 1 subjects was high at 60.0%, consistent with rates of 69.8% reported from South Africa[4], 75% from Tanzania[5], and 77.1% from Nigeria[6]. Community and health professional awareness on the presentation of diabetes in young people is warranted given this late presentation and the likely substantial numbers of

deaths where the correct diagnosis is not made at all. Type 1 patients were generally lean or underweight at diagnosis, and presented with classic symptoms. There was a female preponderance as is often observed in low-incidence countries[18].

There was also a female preponderance in the type 2 population, consistent with data in adults in Kumasi[19]. Type 2 subjects were often overweight. This is of concern, as overweight is now not uncommon in Ghanaian children and youth[20,21]. All had acanthosis nigricans - a physical marker suggestive of insulin resistance[22]. Interestingly, seven of 16 type 2 subjects presented in DKA, suggesting a diagnosis of ketosis-prone type 2 diabetes, which is well-reported in populations in Africa and of African descent[23,24].

The youngest child was 10 mo of age at diagnosis. Development of diabetes at a young age can indicate a monogenic cause, and genetic testing is indicated if the onset is < 6 mo of age or if there are syndromal features of known single-gene defects (which were not present in the infant in this series)[25]. In some of these cases, alternate non-insulin therapy may be possible[25].

Even with accurate diagnosis, mortality has been high in studies in sub-Saharan Africa[26-28], but there are indications it is falling - for instance in Rwanda it was found to be between 13.9-40.2 per 1000 patient years, depending on the fate of those lost to follow-up[29]. The figure of 32/1000 from this study is in this range - and in Rwanda like in Ghana, care is improving as supplies are made available[30]. This improvement in survival is seen in the dramatic increase in the clinic population from 23 to 102 cases over the five years - "if you build it they will come" - and not just come but survive and thrive. Such rapid increases in numbers in clinics that are able to provide standard care (also seen in Tanzania[31]) indicate the strain that will be on resources as survival improves as insulin and other critical supplies are provided by programs such as IDF Life for a Child, and paediatric endocrinologists, trained in Kenya[32] and elsewhere, return to their home countries to establish clinics.

Patient education is critical in improving care. At this study clinic, all patients are called on the telephone to come in for education every fortnight. They are taught at an appropriate educational level about the pathophysiology of diabetes, how to appropriately store and administer insulin, injection sites, adjust doses, manage diet and exercise, detect acute complications, *etc*.

The study results demonstrate the socio-economic challenges faced by many subjects, and the necessity for support with supplies, consistent with past reports[1-3,28]. A number of young people were also facing challenges with continuing their education, as demonstrated in the study by Kratzer[2].

Some families do not have access to home refrigeration for insulin storage, and so place the insulin in a clay pot using evaporative cooling. Such methods do substantially reduce storage temperatures unless humidity is very high[33].

Limitations

The major limitation of this study is the lack of ability and resources to measure autoantibodies and C-peptide to confirm the diagnosis of type 1 or type 2, or an atypical form. Such assistance with typology would not only be interesting scientifically, but would be helpful to individualise management. However, the presence or absence of autoantibodies alone may not be categorical in this population. Agyei-Frempong *et al*[34] in a study of autoimmunity in a population of adults with diabetes in Kumasi found that glutamic acid decarboxylase (GAD) antibody and/or insulinoma antibody (IA2) were present in 35% of those on insulin and 16.5% of those not requiring insulin.

Summary and recommendations

In summary, both type 1 and type 2 diabetes occur in young people in northern Ghana, with high rates of DKA at onset, and a female preponderance. Deaths in the first few years are still not uncommon. Community and health professional awareness is indicated to achieve prompt and accurate diagnosis and prevent deaths at onset. Although not assessed in this study, it is reasonable to conclude that further health professional and patient education is needed to continue to improve management, and therefore reduce the risk of long-term complications. Improvements in the availability of diagnostic technology (particularly blood glucose meters and strips) is also indicated. A patient support group would also be very beneficial.

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COMMENTS

Background

Limited information is available on types of diabetes in young people in Africa, nor on prognosis.

Research frontiers

The epidemiology and prognosis of diabetes in young people in sub-Saharan Africa is of importance as services are developed to look after these young people.

Innovations and breakthroughs

This study shows that both type 1 and type 2 diabetes are occurring in young people in Ghana, with some phenotypic overlap. Mortality was found to be 32.2 per 1000 patient years.

Applications

The study shows how numbers of children and young people in a clinic in a less-resourced country quickly grow as care is given in a paediatric endocrine clinic.

Peer-review

This study offers a valuable insight in the clinical profile of diabetes in population of children and adolescents in Ghana. The subject is interesting and worth investigating, since the data regarding diabetes burden in Africa are still scarce and the study population is particularly vulnerable.

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ORIGINAL ARTICLE

Retrospective Study

Eye and foot checks in patients with diabetes on haemodialysis: Are they done, and who does them?

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Author contributions: Mothojakan NB and Hussain S undertook the research and data analysis; Mothojakan NB wrote the first draft of the manuscript; McCafferty K and Yaqoob MM instigated the research and reviewed the manuscript; Chowdhury TA reviewed the manuscript and undertook all revisions and is the guarantor.

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Abstract

AIM

To determine if retinal and foot checks are carried out on patients with diabetes receiving haemodialysis.

METHODS

Eighty-four patients with diabetes receiving haemodialysis were asked if they recalled having eye and foot screening in the last year, and if so, by whom was the check done.

RESULTS

Seventy-seven (91.7%) patients recalled having an eye check in the preceding 12 mo. Of these, 52 (67.5%) did so in an ophthalmology clinic, 17 (22%) in retinal screening, three (3.9%) in an optician clinic. Three patients (3.9%) went to both ophthalmology and retinal screening, and two (2.6%) attended an ophthalmology and optician. Seventy (83.3%) patients recalled having a foot check in the preceding 12 mo. Of these, 33 (47.1%) were done by practice nurse, 14 (20%) by a diabetes nurse, 11 (15.7%) by a general practitioner, eight (11.4%) by a chiropodist, and four (5.7%) were each checked by renal nurse, diabetes consultant, junior doctor, or unknown person at a foot clinic.

CONCLUSION

Most patients with diabetes on haemodialysis are able to recall having an eye check in the last year, although 8.3% could not. A significant proportion of patients could not recall having a foot check (16.7%) in the last year. This baseline audit suggests that an improvement in the rate of foot screening is important to achieve in patients with diabetes on haemodialysis in our unit.

Key words: Diabetes; Haemodialysis; Foot screening; Retinal screening

Core tip: Diabetes is the commonest cause of end stage renal failure in many countries. Patients with diabetes on haemodialysis are at high risk of retinal and foot problems, and need regular screening to ensure they do not develop problems related to these complications. Our survey suggests that most patients are getting eye checks, but a significant number are not getting foot checks. This is an important area for all dialysis units to consider. We recommend that patients have foot screening whilst on dialysis, which may require further training for dialysis nurses.

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INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal failure in the United Kingdom[1]. Screening programmes enable detection of early changes associated with microvascular complications of diabetes, including diabetic retinopathy and peripheral neuropathy. Many national guidelines recommend that all patients with diabetes have yearly screening of feet and eyes to reduce the risk of blindness and avoidable limb amputations[2,3]. With the increasing prevalence of diabetes, attendance at screening programmes is of the utmost importance in order to reduce the risk of complications.

Patients with diabetes who have end stage renal failure (ESRF) on regular haemodialysis attend hospital for dialysis very frequently, and as a result find it difficult to attend other appointments. We have previously noted poor attendance to other clinics and appointments in patients on haemodialysis. East London is an area of high social deprivation, and many patients are elderly, with multiple co-morbidities, whose first language is often not English, and these factors may affect their ability to access healthcare[4]. Patients with diabetes on haemodialysis are at particular risk of foot and eye problems[5]. Microvascular complications of diabetes arise due to poor glycaemic control, and indeed haemodialysis patients with poor glycaemic control have been found to have poorer survival compared to those with good glycaemic control[6].

Recent United Kingdom guidelines highlight the need for annual foot and eye screening for patients with diabetes on haemodialysis[4]. The aim of this study was to determine if patients with diabetes on our haemodialysis unit could recall having retinal screening and foot surveillance in the past year, and to find out who had performed this.

MATERIALS AND METHODS

This retrospective study was carried out on the dialysis unit of the Royal London Hospital, a tertiary centre which serves a large cohort of renal patients in East London, United Kingdom. A brief questionnaire was designed for patients with diabetes receiving dialysis, asking whether patients recalled having "a diabetes eye check" or "diabetes foot check" in the past 12 mo. Patients who had received a diabetes eye check were asked where this had taken place: At an optician, eye clinic, retinal screening service or elsewhere. Patients who had a diabetes foot check where asked who had performed the procedure; a doctor, diabetes nurse, renal nurse or podiatrist.

Participants were recruited to the study from August to September 2015, whilst receiving haemodialysis on the renal unit. Inclusion criteria for the study included: Patient currently receiving haemodialysis, patient was diagnosed with diabetes for at least a year and able to receive care in the community. Patients were excluded from the study if they had communication difficulties.

All statistical analysis and graphs were performed using GraphPad Prism 7 (GraphPad software inc, California, United States) software. Quantitative data were expressed as frequencies or mean ± SD as appropriate. Qualitative data were expressed as frequencies.

RESULTS

Patient characteristics

Eighty-four patients met the inclusion criteria and agreed to participate in the study. Patient characteristics are shown in Table 1. Sixty point seven percent of the participants were male and 39.3% were female. The mean age of the cohort was 63.9 ± 10.35 years. Insulin only therapy was used by 53.6% of the participants. The remaining participants were diet-controlled (11.9%), on medication only (19%) or medication and insulin (15.5%).

Eye checks

Figure 1 shows eye check uptake in the patients

Table 1 Demographic characteristics of patients surveyed *n* (%)

Variables	Patients
Gender	
Male	51 (60.7)
Female	33 (39.3)
Age (mean ± SD)	63.9 ± 10.35
Ethnicity	
African - Caribbean	38 (45.2)
Asian - Bangladeshi	22 (26.2)
Asian - Indian	4 (4.8)
Asian - Pakistani	2 (2.4)
Asian - Other	4 (4.8)
White - British	9 (10.7)
White - Other	2 (2.4)
Other	3 (3.6)
Treatment regimen	
Diet only treated	10 (11.9)
Oral hypoglycaemic only treated	16 (19.0)
Insulin + oral hypoglycaemic treated	13 (15.5)
Insulin only treated	45 (53.6)

surveyed. Seventy-seven (91.7%) of patients reported having an eye check in the last 12 mo. Of these, 52 (67.5%) did so in an ophthalmology clinic, 17 (22%) in retinal screening, three (3.9%) in an optician, three (3.9%) went to both ophthalmology and retinal screening, and two (2.6%) attended an ophthalmology and optician.

Diabetic foot screening

Figure 2 shows foot check uptake in the patients surveyed. Seventy (83.3%) patients recalled having a foot check in the previous 12 mo. Of these, 33 (47.1%) were carried out by a practice nurse, 14 (20%) by a diabetes specialist nurse, 11 (15.7%) by the general practitioner (GP), eight (11.4%) by a chiropodist, and four (5.7%) had been a renal nurse, a diabetes consultant, a junior doctor, or unknown person at a foot clinic.

DISCUSSION

Screening programmes have an important role in the prevention and early detection of retinopathy and neuropathy. We are unaware of any previous studies investigating the uptake of screening programmes in haemodialysis patients. In the United Kingdom in 2012-2013, 80.2% of patients offered diabetic eye screening attended. Recent recommendations suggest that is should be possible for a minimum of 85% of those offered digital retinal screening to attend. Screening uptake in 2012-2013 was lower than the results of our study, where we found that 91.7% of patients attended an eye check in the previous last year, suggesting that patients with diabetes on haemodialysis are aware of the need to undertake eye checks on a regular basis.

The United Kingdom National Diabetes Audit 2015-2016, found that 86.7% of patients with diabetes had foot surveillance that year[7], which was slightly

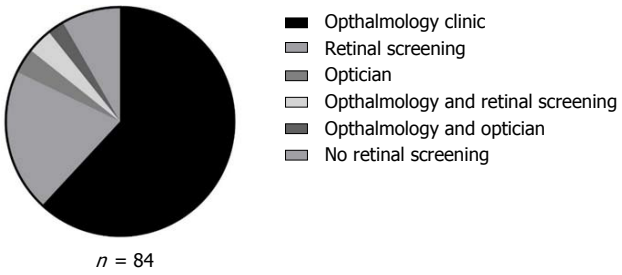

Figure 1 Retinal screening in the cohort.

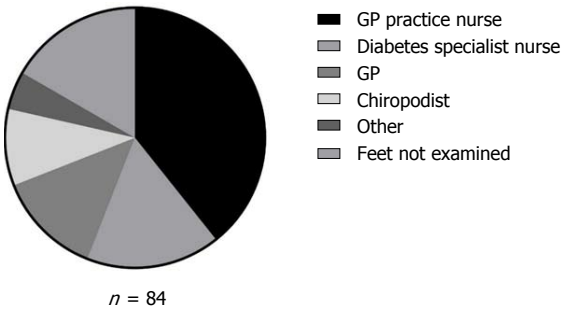

Figure 2 Diabetic foot checks in the cohort. GP: General practitioner.

higher than in our patient survey (83.3%). This is of some concern, particularly as patients with diabetes on haemodialysis are at high risk of foot problems. Recent guidelines recommend that patients have their feet screened every 3 mo with a locally agreed tool, and by staff on the dialysis unit[4].

In the United Kingdom, co-ordination of screening programmes for eyes and feet are led in the community by primary care health professionals. Retinal screening programmes are locally commissioned within Clinical Commissioning Group, and call and recall is organised by review of primary care records. Most retinal screening occurs *via* the retinal screening programme, although patients with established significant retinopathy may attend a medical retinal clinic as well. In the present study, it was found that the ophthalmology clinic was the most common place for eye checks, accounting for 67.5% of all patients. This is unsurprising as many patients on haemodialysis also have other microvascular complications such as retinopathy. A small proportion of patients had eye checks carried out by an optician, which, whilst useful, means that such patients may not be accessing a formal retinopathy screening programme. Interestingly, 7.1% of patients had an eye check carried out more than once in the past year, suggesting some duplication.

Foot checks for people with diabetes are generally performed by trained clinical staff in the primary care centre, which is often the practice nurse. Our study confirmed that nurses in the community were the most common group to carry out diabetic foot checks, with 67.1% of foot checks carried out by the nurses in primary care. Very few patients stated that their feet had ever been examined on the dialysis unit during

dialysis. Patients on haemodialysis have logistical difficulties that make it difficult for them to attend appointments elsewhere. Perhaps this may account for patients missing screening appointments. A lack of co-ordination between the health care professionals caring for the patients may have also resulted in missed screening opportunities, as it is assumed that they have been carried out elsewhere. Patients spend significant amounts of time on dialysis, and this may provide an excellent opportunity for screening of feet and eyes to be undertaken opportunistically, as well as reducing the need for patients to attend hospital in between dialysis sessions. This is specifically mentioned as an important aim in recent United Kingdom guidelines, and clearly needs to be addressed in our haemodialysis unit[4]. These guidelines recommend that annual checks are documented, and made available to all those involved in the care of these patients.

The introduction of a robust system of documentation, would ensure that individuals involved are aware of recent checks and when they last took place, avoiding unnecessary duplication. Furthermore, access to a named link worker on the dialysis unit who would ensure that screening is carried out, which could ensure that patients have received eye and foot screening, and are also educated in looking for early signs of significant foot problems, and highlight these to health professionals at an early stage. Inter-professional learning between diabetes and renal specialists may facilitate improvements in care.

There are some limitations to this study, including a small patient cohort and the fact that it was carried out at a single tertiary centre. Patients with communication difficulties were excluded from the study, and it is possible that this group of patients may have had difficulty accessing healthcare, and may also be more likely to miss screening appointments. The study did not examine the barriers to patients attending screening appointments.

Patients with diabetes on dialysis are at risk of microvascular complications, and due to logistical issues have difficulties attending other appointments. Most patients had an eye check in the last year, with a lower percentage of recalling a foot check in the last year. It is hoped that the introduction of recent guidelines will improve the uptake of screening.

COMMENTS

Background

Patients with diabetes on haemodialysis are at high risk of diabetes complications including foot and eye problems. It is not known whether patients with diabetes on haemodialysis attend regular screening appointments for foot and eye checks. This survey aimed to determine this information.

Research frontiers

It is increasingly recognised that prevention of diabetic complications in patients on haemodialysis is important. At the moment, it is unknown whether improving glucose control or other risk factors will reduce morbidity and mortality in such patients.

Innovations and breakthroughs

Recent United Kingdom guidelines suggest a more proactive approach to managing patients with diabetes on haemodialysis. It is hoped that with more structured care, better outcomes will be seen.

Applications

The authors show that most patients with diabetes on haemodialysis attend for eye checks, but that foot checks may be neglected. The authors propose that foot checks on dialysis would be an effective way to ensure proactive management of foot problems in patients on dialysis.

Peer-review

Mothojakan *et al* report the findings of a retrospective study of whether foot and eye screening is being done on diabetic patients undergoing hemodialysis. The paper has been revised in light of a previous review and is well written, easy to follow and without any obvious errors or unfounded claims.

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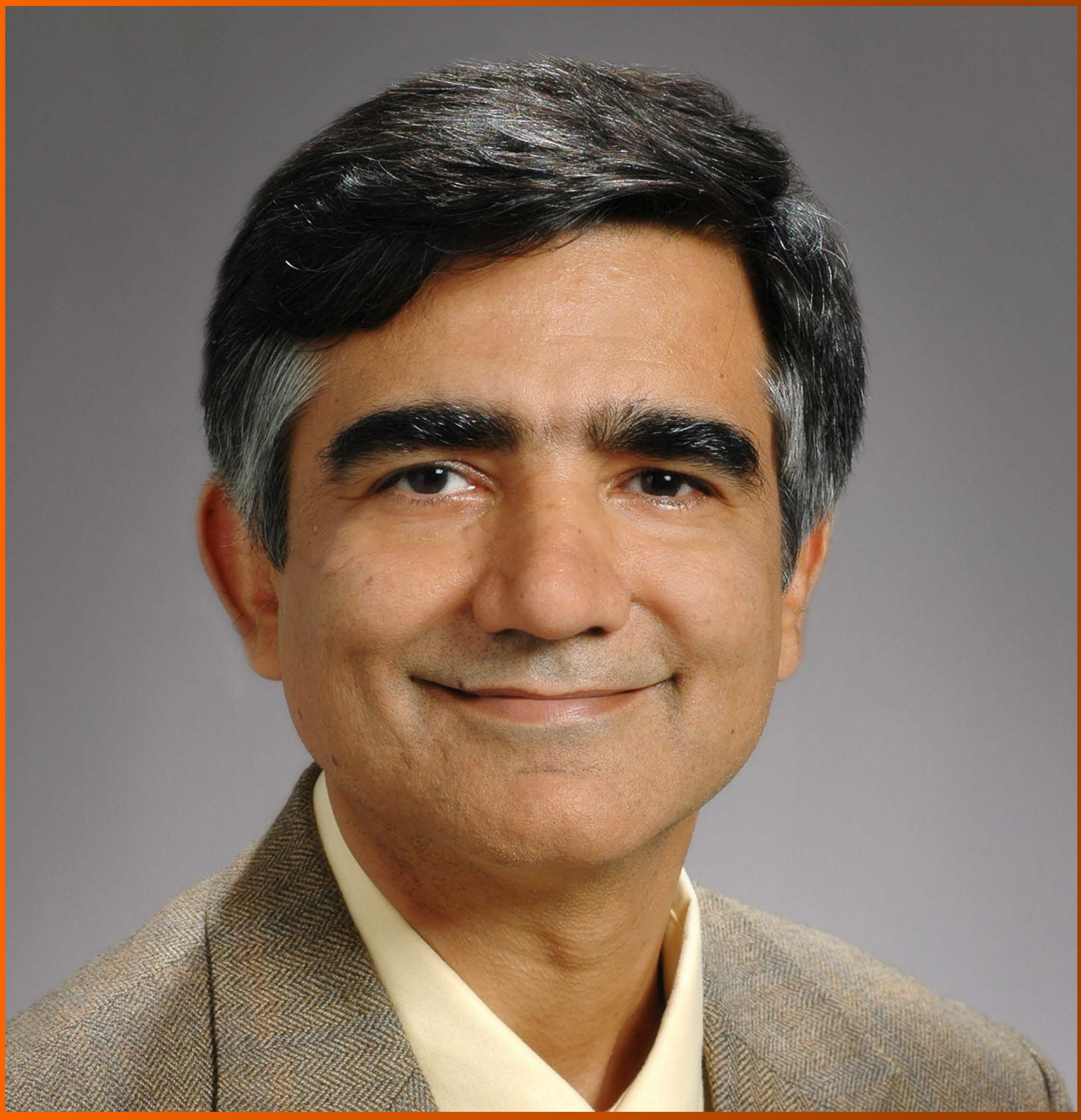

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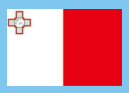

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REVIEW

Assessing the evidence for weight loss strategies in people with and without type 2 diabetes

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Abstract

This review will examine topical issues in weight loss and weight maintenance in people with and without diabetes. A high protein, low glycemic index diet would appear to be best for 12-mo weight maintenance in people without type 2 diabetes. This dietary pattern is currently being explored in a large prevention of diabetes intervention. Intermittent energy restriction is useful but no better than daily energy restriction but there needs to be larger and longer term trials performed. There appears to be no evidence that intermittent fasting or intermittent severe energy restriction has a metabolic benefit beyond the weight loss produced and does not spare lean mass compared with daily energy restriction. Meal replacements are useful and can produce weight loss similar to or better than food restriction alone. Very low calorie diets can produce weight loss of 11-16 kg at 12 mo with persistent weight loss of 1-2 kg at 4-6 years with a very wide variation in long term results. Long term medication or meal replacement support can produce more sustained weight loss. In type 2 diabetes very low carbohydrate diets are strongly recommended by some groups but the long term evidence is very limited and no published trial is longer than 12 mo. Although obesity is strongly genetically based the microbiome may play a small role but human evidence is currently very limited.

Key words: Protein; Glycemic index; Very low calorie diet; Very low carbohydrate diet; Low fat diets; Intermittent energy restriction; Alternate day fasting

Core tip: Very low energy or very low calorie diet (VLCD) may reverse early type 2 diabetes and very low carbohydrate diets may offer a short term advantage in reducing medication use and/or lower HbA1c more than a more conventional diet. Intermittent energy restriction may be helpful in some people but more data is required. Long term weight maintenance after VLCD may be helped by a higher protein lower glycemic index diet but drugs and partial meal replacements are also helpful.

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OBSERVATION COHORTS

Observational cohorts from the Nurses' Health Study Ⅰ and Ⅱ and the Health Professionals Follow up study with a total of 120000 participants have been very useful at examining dietary predictors of weight gain[1,2]. In these cohorts there was a weight gain of 1.45 kg over 4 years. A one cup increase in: (1) sugar sweetened beverages increased weight gain by 0.36 kg; and (2) fruit juice by 0.22 kg while a 1 cup increase in coffee decreased weight by 0.14 kg as did tea by 0.03 kg. Substituting water for sugar sweetened beverages decreased weight gain by 0.49 kg. Greater than average increase in weight was associated with potatoes and French Fries, sugar-sweetened beverages, red meat, alcohol, TV watching, short or long hours of sleep (< 6 or > 8 h/night) and quitting smoking. Lower than average weight gain was associated with a high consumption of vegetables, whole grains, fruit, nuts, yogurt and physical activity.

ISSUES FOR WEIGHT LOSS AND WEIGHT MAINTENANCE

Long term caloric reduction and weight loss induces a reduction in resting metabolic rate that is usually greater than expected by the lean tissue loss[3], and increased energy efficiency of digestion and absorption and movement[4] all of which make weight maintenance a difficult proposition. Hunger is increased and appetite and satiety hormones still deranged 12 mo after initial weight loss despite weight stability or even some weight regain[5]. Whether the higher thermic effect[6] and higher satiety value of protein[7] helps maintain weight loss is not totally clear. Higher fiber intake and lower energy density plus increased polyunsaturated fat intake have been associated with better weight maintenance[8]. Long term weight maintenance after large weight losses in the National Weight Control Register is associated with frequent self-monitoring of body weight and food intake, consistency of food intake, always eating breakfast, low variety of food, low fat, low fast food intakes and high levels of regular physical activity (10-11 mJ/wk) although none of these behaviors may be causally related to weight maintenance Once these successful maintainers have maintained a weight loss for 2-5 years, the chances of longer-term success greatly increase[9,10].

LOW FAT DIETS

Low fat ad libitum diets have been recommended for many decades on the basis of several observations: (1) energy from fat is less satiating than energy from carbohydrate, and a high fat/carbohydrate ratio (and thus higher energy density) in the diet can promote passive overconsumption, a positive energy balance and weight gain in susceptible individuals as most individuals eat a fixed volume of food[11-13]; (2) fat is more readily absorbed from the intestine than carbohydrate and faecal energy loss is much lower with a high dietary fat/carbohydrate ratio; (3) carbohydrate is more thermogenic than fat[14] and energy expenditure is lower during positive energy balance produced by a diet with a high fat/carbohydrate ratio than during positive energy balance produced by a diet with a low fat/carbohydrate ratio[15]; and (4) a high fat diet may damage the intestinal barrier and cause intestinal dysbiosis[16,17].

Low fat diets were reviewed many years ago by Astrup *et al*[18]. Summaries for all the diets are found in Table 1. He found that low-fat diets cause weight loss proportional to pretreatment body weight and weight loss is correlated positively to the reduction in dietary fat content. A reduction of 10% fat energy produces an average 5-kg weight loss in obese persons. After major weight loss, an ad libitum low-fat diet program appeared to be superior to caloric counting in maintaining the weight loss 2 years later. A recent meta-analysis from Tobias *et al*[19] found low diets were not different to high fat weight loss diets but worth 5 kg compared with no intervention. A Cochrane meta-analysis from Hooper confirmed the weight loss effects of a low fat diet compared with usual diet with an effect size of 1.5 kg[20].

HIGH PROTEIN DIETS

High protein weight loss diets reduce the intake of carbohydrate and fat but maintain protein intake to take advantage of their greater satiety (10%-15% less food intake after a protein preload[21]) and thermic effects. Atkins and South Beach diets maintain protein intake but in addition dramatically reduce carbohydrate and replace it with fat. Omitting a major food group inevitably leads to weight loss but long term adherence is difficult.

Clifton *et al*[22] performed a meta-analysis of planned high protein diets *vs* normal protein weight loss diets with at least 10% protein difference planned or expected (*e.g.*, Atkins diets) and followed up for 12 mo or more. The actual reported difference in protein intake at the end of the study was usually 2%-5% of energy. Thirty-two studies with 3492 individuals were analyzed with data on fat and lean mass, glucose and insulin data was available from 18 to 22 studies and lipids from 28 studies. This meta-analysis included the large but very negative Sacks study[23]. A difference in favor of the high protein of about 0.4 kg for weight and fat mass was found. A difference of 5% or greater in percentage protein between diets at 12 mo was associated with a 3-fold greater effect size compared with < 5% (P = 0.038) in fat mass (0.9 *vs* 0.3 kg). Fasting triglyceride and insulin were also lower with high protein diets.

Table 1 Weight loss diets in people without diabetes

Type of diet	Type of summary document	Effect size	Long term data	Recommendation	Risk markers
Low fat diet	Systematic review[18]	10% reduction in fat lowers weight by 5 kg			
Low fat diet	Meta-analysis[19]	Not different to high fat weight loss diets			
Low fat diet	Cochrane[20] meta-analysis 32 RCT, 54000 participants At least 6-mo duration	Worth 5 kg compared with control Mean reduction 1.5 kg for low fat without intention to lose weight	No reduction with time	High quality evidence-effect seen in almost all studies	
Conclusion				A useful strategy well worth pursuing	
High protein diet	Meta-analysis of 12 m or greater weight loss studies 3492 individuals[22]	SMD 0.14 for weight $P = 0.008$) and 0.22 for fat mass, $P < 0.001$ for 2%-5% energy differences in protein. > 5% energy protein difference 0.9 kg weight loss	Data out to 5 yr still shows a small residual effect		Lower triglyceride (SMD 0.17, $P = 0.003$) and lower insulin (SMD 0.22, $P = 0.042$)
High protein diet	Meta-analysis of controlled short term studies[24]	0.79 kg weight 95%CI: -1.50, -0.08 kg), 0.8 kg greater fat mass loss (-0.87 kg; 95%CI: -1.26, 0.48), 0.43 kg (95%CI: 0.09, 0.78) reduction in lean loss			Lower triglyceride (-0.23 mmol/L; 95%CI: -0.33, -0.12 mmol/L). Reductions in falls in REE (595.5 kJ/d; 95%CI: 67.0, 1124.1 kJ/d)
Conclusion				Small effects. Difficult to maintain a higher protein intake long term as other sources of calories creep in	
Very low carbohydrate diets	Energy controlled < 45% CHO *vs* < 30% fat 23 trials 2788 participants[31]	Weight outcomes same			Slightly lower LDL, TG, increased HDL
Very low carbohydrate diets	Meta-analysis of 6 mo studies, 11 studies[25]	Atkins diet better by WMD -2.17 kg; 95%CI: -3.36, -0.99	Not long term	No long term benefit, possible adverse CVD effects	Triglyceride was lowered WMD -0.26 mmol/L; 95%CI: -0.37, -0.15 by the low carbohydrate diet; LDL elevated by WMD 0.16 mmol/L; 95%CI: 0.003, 0.33). HDL elevated WMD 0.14 mmol/L; 95%CI: 0.09, 0.19
Very low carbohydrate diets	Meta-analysis of 12 mo or > studies, $n = 5$[25]	Weight outcomes same		No long term benefit	
Conclusion				No long term benefit	
Very low calorie diet	Review of 12 studies[35] of VLCD *vs* behavioural program and diet change	VLCD was worth an additional 3.9 kg at 12 m and 1.4 kg at 24 m and 1.3 kg at 38-60 m. Dropouts were the same at 19%-20% which was lower than expected	Long term benefit seen	Worth trying with weight loss maintenance programs	
Very low calorie diet	Single hospital based clinic $n = 1109$[36]	19% still attending at 3 yr and the mean weight loss of this group was 6.4 kg. Weight loss was 7.7% *vs* 2.3% for drugs (topiramate plus phentermine or sibutramine) compared with no drugs			
Conclusion				Well worth trying if large weight loss required	
Weight maintenance after VLCD	8 European centres[38] 11% weight loss with VLCD after 8 wk Randomised to high or normal protein 25% *vs* 13% and high or low GI 15U different	Fewer participants in the high-protein and the low glycemic-index groups than in the low-protein–high-glycemic-index group dropped out of the study (26.4% and 25.6% *vs* 37.4%; $P = 0.02$ and $P = 0.01$)	The difference in weight regain after 1 yr[39] between protein groups was 2.0 (0.4, 3.6) kg ($P = 0.017$) (completers analysis, $n = 139$) or 2.8 (1.4, 4.1) kg ($P < 0.001$) (intention-to-treat analysis, $n = 256$)	In the shop centres (where food was provided) protein had a more powerful effect (2.7 kg compared with low protein, $P < 0.001$) while low GI had less effect (0.48 kg, NS)	

				Protein may have modest long term weight maintenance effects	
Weight maintenance after VLCD	189 participants on VLCD for 3 mo then high or normal protein for 12 mo[40]	No difference between diets Weight regain over 9 mo was modest at 2 kg with a final weight loss of 14.5 kg overall. Overall dropout rate was 53% and compliance measures to the high protein diet were limited		Because compliance measures were limited conclusions on benefit (or absence of benefit) are limited	
Conclusions				Protein may be of some benefit, GI isn't long term. More trials required	
Intermittent energy restriction	2 d partial fast and 5 normal days or alternate day fasting	Weight loss similar to CER over 3-6 mo[40-42,44,45]	No long term data		No additional metabolic benefit[47,48]
Conclusion				Insufficient data, no long term data. More work required	
Glycemic index	23 young adults[50] low GI ad lib *vs* Low fat diet with energy reduction of 250-500 kcal	Weight loss 7.8% *vs* 6.1% (NS)			Triglyceride was lowered by 37.2% and 19.1% (P = 0.005) at 6 mo with no difference at 12 mo. PAI-1 was lowered by 39% with the low GI diet *vs* a 33% rise (despite the weight loss)
Glycemic index	73 young adults low gIycemic load diet *vs* low fat diet[51]	No difference at 6, 12, 18 mo Insulin above the median (57.5 μIU/mL; n = 28) at 30 min of OGTT -5.8 *vs* -1.2 kg on low GL diet *vs* low fat diet (P = 0.004) and body fat percentage (-2.6% *vs* -0.9%; P = 0.03). No difference in insulin sensitive group			CVD risk markers the same
Conclusion				Insufficient data for any conclusions	
Mediterranean diet	Mediterranean *vs* low fat *vs* low carbohydrate diet in 322 people in a workplace setting[51]	Weight loss in the 272 completers was 2.9 kg for the low-fat group, 4.4 kg for the Mediterranean-diet group, and 4.7 kg for the low-carbohydrate group; a moderate reduction only (P < 0.001 for the interaction between diet group and time)	During 6 follow-up period, participants had regained 2.7 kg of weight lost in the low-fat group, 1.4 kg in the Mediterranean group, and 4.1 kg in the low-carbohydrate group (P = 0.004 for all comparisons) For the entire 6-yr period, the total weight loss was 0.6 kg in the low-fat group, 3.1 kg in the Mediterranean group, and 1.7 kg in the low-carbohydrate group (P = 0.01 for all comparisons) with the Mediterranean group and the low-carbohydrate group not different from each other (P = 0.22)[52]		
Conclusion				Mediterranean diet best long term and has the longest follow up along with VLCD	

Low sugar diet	Meta-analysis of 30 trials and 38 cohorts[53]	Adults decrease in body weight (0.80 kg, 95%CI: 0.39 to 1.21; $P < 0.001$) Cohort studies sugar caused increase weight increase of 0.75 kg, 95%CI: 0.30 to 1.19; $P = 0.001$) Interventions in children SSB *vs* control beverage 1 kg (95%CI for the difference, -1.54 to -0.48)[54]	12 mo difference in weight of 1.9 kg SSB *vs* water disappeared 12 mo after trial stopped[55]	
Conclusion				Strong evidence for the benefit of sugar reduction in beverages
Multicomponent	33 RCTS of at least 1 yr's duration[56]	Weight loss *vs* exercise 3.2 kg, 95%CI: -4.8 kg to -1.6 kg) Type of diet not important	Low-fat diets, some with meal replacements, with physical activity and behavior change training gave most effective long-term weight change in men (-5.2 kg after 4 yr)	
Multicomponent	Commercial weight loss programs[57]	Pooled results from five study arms in commercial weight management programs showed significant weight loss at 12 mo (-2.22 kg, 95%CI: -2.90 to -1.54) Two commercial weight loss arms (mean difference -6.83 kg, 95%CI: -8.39 to -5.26) GP interventions mean difference -0.45 kg, 95%CI: -1.34 to 0.43)		
Conclusion				Commercial plans of some value
Calcium	Meta-analysis of calcium RCTs	RCTs of about 600 overweight and obese individuals from 7 trials dietary calcium supplementation of about 1000 mg was associated with weight loss and fat loss of approximately 1 kg over 6 mo and had a greater effect in pre - than in postmenopausal women[59]	Calcium (1000 mg) and vitamin D after 3 yr of follow-up women with daily calcium intakes of < 1200 mg at baseline on supplements were 11% less likely to experience weight gain[61]	
Conclusion				Marginal effect only
Dairy	Meta-analysis of 27 trials of dairy added to energy restriction[62] Meta-analysis of added calcium or dairy without weight restriction-no effects seen[60]	A greater reduction in body weight [-1.16 kg (95%CI: -1.66 to -0.66), $P < 0.001$, $I^2 = 11\%$, QR = high, $n = 644$) and body fat mass [-1.49 kg (95%CI: -2.06 to -0.92), $P < 0.001$, $I^2 = 21\%$, $n = 521$, QR = high) smaller loss of lean mass of 0.36 kg (0.01, 0.71 kg), $P = 0.04$, $I^2 = 64\%$, $n = 651$, QR = moderate)	No long term data	
Conclusion				Dairy may be useful component of a weight loss diet but does nothing by itself in the absence of weight loss

CER: Continuous energy restriction; CHO: Carbohydrate; GI: Glycemic index; PAI-1: Plasminogen activator inhibitor-1; QR: Quality rating; RCT: Randomised control trial; SMD: Standardized mean difference; VLCD: Very low calorie diet; WMD: Weight mean difference.

Other lipids and glucose were not different. A meta-analysis of short term calorie controlled interventions was performed by Wycherley *et al*[24]. Despite the similar energy prescription weight loss was greater on the high protein, low fat diet with a difference in weight of -0.79 kg and fat mass of 0.8 kg with lower triglycerides. There

was also mitigation of reductions in fat-free mass of 0.43 kg and resting energy expenditure.

There have been several meta-analysis of low carbohydrate diets[25-31]. One compared low carbohydrate diets (< 45%) *vs* low fat (< 30%) diets in an energy controlled, constant protein design. In 23 trials containing 2788 participants weight outcomes were the same with slightly lower low-density lipoprotein (LDL), increased high-density lipoprotein and lower TG[31]. In a meta-analysis of 5 studies[25] of 12 mo or more duration there was no difference in weight although 11 studies of 6 mo or more duration[25] showed a 2 kg difference in favor of the Atkins diet. Although triglyceride was lowered as expected by 0.35 mmol/L by the low carbohydrate diet LDL cholesterol was still elevated by 0.2 mmol/L by the high saturated fat diet which could increase the risk of cardiovascular disease (CVD) suggesting the Atkins diet may not be the best diet for those at risk of CVD[25,32-34]. Flow mediated dilatation which is a reasonable proxy for CVD risk is impaired after an Atkins diet despite weight loss and blood pressure and glucose reduction[35]. South Beach style diets which use unsaturated fats instead may be better for those at risk of CVD[34].

MEAL REPLACEMENTS AND VERY LOW CALORIE DIETS

Another variant of a high protein diet is the meal replacement which provides mostly protein with a small amount of carbohydrate or fat but also provides a very structured, controlled intake especially in its very low calorie diet (VLCD) form. The latter is not frequently used because of rapid weight regain after its cessation but if drugs are used better weight maintenance can be achieved.

A recent review examined 12 studies with 974 participants comparing VLCD to behavioural programs that would be conducted in a medical clinic. Compared with behavioural programs (mostly diet alone) VLCD was worth an additional 3.9 kg at 12 m and 1.4 kg at 24 m and 1.3 kg at 38-60 m. Dropouts were the same at 19%-20% which was lower than expected[36]. A follow up of an obesity clinic hospital population of 1109 hospital patients given VLCD showed that 19% were still attending at 3 years and the mean weight loss of this group was 6.4 kg. Weight loss was 7.7% *vs* 2.3% for drugs (topiramate plus phentermine or sibutramine) compared with no drugs[37].

WEIGHT MAINTENANCE AFTER VLCD

Larsen *et al*[38] completed a large pan European trial in 8 centres which randomised participants to a normal or high protein diet or a low glycemic index or moderate glycemic index. After 773 completed the VLCD phase they were randomised to the maintenance diets for 6 mo. Although the high protein diet was planned to be 25% of energy compared with 13% in the normal diet the difference between the two was only 5%. The GI was planned to be 15 U different but only a 5 U difference was achieved. In an intention-to-treat analysis, the weight regain was 0.93 kg less in the high-protein group than in the low-protein group (P = 0.003) and 0.95 kg less in low-GI diet than in the high GI diet (P = 0.003). Only the low protein, low GI group gained a significant amount of weight over the 6 mo (1.67 kg; P < 0.01). The follow up was extended to 1 year in 2 of the centres. The difference in weight regain after 1 year between protein groups was 2.0 kg (P = 0.017). No consistent effect of GI on weight regain was found[39].

Contrary results were found by Delbridge *et al*[40] who placed 180 participants on a VLCD for 3 mo and then randomised them to a high protein weight maintenance diet or a normal protein diet. Weight regain over 9 mo was modest at 2 kg with a final weight loss of 14.5 kg overall. Overall dropout rate was 53% and compliance measures to the high protein diet were limited so it is difficult to draw any firm conclusions form this study.

ALTERNATIVE APPROACHES TO FULL VLCD

Intermittent energy restriction consists of either 2 d of 600-880 kcal/d with 5 d of a normal diet or alternate day fasting. The weight loss results are very similar to a 25%-30% calorie reduction every day over 3-6 mo[41,42]. Similar results have been seen with alternate day fasting[43] and week on/week off diets[44] and there is some evidence of usefulness in people with type 2 diabetes[45,46]. Alternate day fasting may be just as efficacious as full VLCD[47]. The suggestion there may be metabolic benefit of intermittent energy restriction is currently unproven[48,49].

GLYCEMIC INDEX

There are very limited studies for weight loss in people without diabetes. Ebbeling *et al*[50] studied 23 young obese adults over 12 mo comparing an ad libitum low GI diet to a low fat diet with an energy reduction of 250-500 kcal/d. Body weight was lowered by a similar amount at 12 mo. Plasminogen activator inhibitor-1 was lowered by 39% with the low GI diet *vs* a 33% rise (despite the weight loss). In a second study of 73 young obese adults a low glycemic load diet was not different from a low fat diet at 6, 12 and 18 mo[51]. For those with a high insulin concentration at 30 min after a 75 g OGTT (*i.e.,* insulin resistant) the low-glycemic load diet produced a greater decrease in weight (-5.8 kg *vs* -1.2 kg; P = 0.004) than the low-fat diet at 18 mo. No differences were seen in the insulin sensitive group. CVD risk markers were not influenced by insulin response status.

MEDITERRANEAN DIET

Shai *et al*[52] compared a Mediterranean to an Atkins and a low fat weight loss diet in 322 subjects with a mean body mass index (BMI) 31 of whom 86% male in

a controlled workplace setting in the Negev desert (The DIRECT study). At 2 years 84.6% were still enrolled in the study. Weight loss in the 272 completers was 2.9 kg for the low-fat group, 4.4 kg for the Mediterranean-diet group, and 4.7 kg for the low-carbohydrate group (a moderate reduction) (only $P < 0.001$ for the interaction between diet group and time). Predictors of successful weight loss at 6 m were increasing the intake of vegetables and decreasing the intake of sweets and cakes.

At 6 years after study initiation, 67% of the participants had continued with their originally assigned diet, 11% had switched to another diet, and 22% were not dieting ($P = 0.36$ for all comparisons). For the entire 6-year period, the total weight loss was 0.6 kg in the low-fat group, 3.1 kg in the Mediterranean group, and 1.7 kg in the low-carbohydrate group ($P = 0.01$ for all comparisons) with the Mediterranean group and the low-carbohydrate group not different from each other ($P = 0.22$)[53].

LOW SUGAR DIETS

Te Morenga *et al*[54] performed a meta-analysis of low sugar diets. In trials of adults with ad libitum diets reduced intake of dietary sugars was associated with a decrease in body weight of 0.80 kg, $P < 0.001$. Isoenergetic exchange of dietary sugars with other carbohydrates showed no change in body weight. In cohort studies increased sugar intake was associated with a weight increase of 0.75 kg, $P = 0.001$). In children a controlled randomised beverage trials of sugar sweetened beverages *vs* artificially sweetened over 18m demonstrated a weight increase of 6.35 kg in the sugar-free group as compared with 7.37 kg in the sugar group[55]. In 223 overweight/obese adolescents home delivery of water and diet beverages in children who were regular consumers of sugar sweetened beverages for 1 year induced changes in weight (-1.9 kg, $P = 0.04$) compared with the control group at 1 year but this disappeared at 2 years[56].

MULTICOMPONENT AND COMMUNITY-BASED INTERVENTIONS

Robertson *et al*[57] examined weight loss studies in men of at least 1 year's duration and 33 RCTs were located which met the inclusion criteria. Reducing diets tended to produce more favorable weight loss than physical activity alone (mean weight difference after 1 year from a reducing diet compared with an exercise program of 3.2 kg). The type of reducing diet did not affect long-term weight loss. A reducing diet plus physical activity and behavior change gave the most effective results. Low-fat reducing diets, some with meal replacements, combined with physical activity and behavior change training gave the most effective long-term weight change in men of 5.2 kg after 4 years.

Hartmann-Boyce *et al*[58] examined multicomponent interventions delivered in a routine clinical practice environment with assessment at 12 mo. Pooled results from five study arms in commercial weight management programs showed significant weight loss at 12 mo of 2.22 kg. Results from two arms of a study testing a commercial program providing meal replacements also showed a significant weight loss of 6.8 kg. In contrast, pooled results from five interventions delivered by primary care teams showed no evidence of an effect on weight. Clearly commercial weight loss programs can be of value.

DAIRY AND HIGH CALCIUM DIETS FOR WEIGHT LOSS

Calcium

Calcium binds fat in the gut so that an additional dietary calcium intake of 1000 mg increases faecal fat excretion by approximately 5 g/d[59] which has the potential to add to weight loss. In a meta-analysis of RCTs of about 600 overweight and obese individuals from 7 trials dietary calcium supplementation of about 1000 mg was associated with weight loss and fat loss of approximately 1 kg over 6 mo and had a greater effect in pre- than in postmenopausal women[60]. Booth *et al*[61] however found no effect in their meta-analysis. Most interventions used low fat milk as fat intake was not different between intervention and control in these studies. Women who received calcium (1000 mg) and vitamin D had a slightly lower weight gain than did those receiving placebo, and after 3 years of follow-up women with daily calcium intakes of < 1200 mg at baseline who were randomly assigned to supplements were 11% less likely to experience weight gain[62].

Dairy

There have been several meta-analyses of the effect of addition of dairy foods to an energy restricted diet. The most recent one examined 27 trials of > 4 wk's duration[63]. Participants consumed between 2 and 4 standard servings/day of dairy food and 20-84 g/d of whey protein compared to low dairy control diets, over a median of 16 wk. A greater reduction in body weight of 1.16 kg, $n = 644$ and body fat mass 1.49 kg, $n = 521$, 90% of whom were women. These effects were absent in studies that imposed resistance training. Dairy intake resulted in smaller loss of lean mass of 0.36 kg. No between study dose-response effects were seen. A previous meta-analysis[61] found no effect of the addition of calcium or dairy on weight, thirty-one with dairy foods ($n = 2091$), and twenty with Ca supplements ($n = 2711$).

DIETS FOR WEIGHT LOSS IN TYPE 2 DIABETES

In this section we will examine the effects of diets not just on weight but on HbA1c as an HbA1c > 7% would

be one of the prime reasons overweight and obese people with diabetes would be recommended to lose weight. Weight stable dietary changes to lower HbA1c will not be examined (Table 2).

LOWER GLYCEMIC INDEX/LOWER GLYCEMIC LOAD DIETS

Although these diets would be recommended predominantly to lower HbA1c they are also used for weight loss. The Canadian Trial of Carbohydrates in Diabetes[64] enrolled 162 people treated by diet alone who were randomly assigned to high-carbohydrate/high-glycemic-index (HGI) diets; high-carbohydrate/low-glycemic-index (LGI) diets or lower-carbohydrate/high-monounsaturated-fat (LC) diets for 1 year. No differences were seen in weight or HbA1c over 1 year but achieved GI differences were small. A second Canadian low glycemic index diet study[65] in 210 participants with type 2 diabetes on hypoglycemic medication showed no differences in weight over 6 mo compared with a high cereal fibre diet although HbA1c was lowered by 0.32%[65].

Franz *et al*[66] examined randomized clinical trials implementing weight-loss interventions in overweight or obese adults with type 2 diabetes with a minimum 12-mo study duration, a 70% completion rate, and an HbA1c value reported at 12 mo. Eight trials compared different diets while 3 compared diets to usual care. Only two study groups reported a weight loss of $\geqslant$ 5%: A Mediterranean-style diet implemented in newly diagnosed adults with type 2 diabetes and an intensive lifestyle intervention implemented in the Look AHEAD (Action for Health in Diabetes) trial. Both included regular physical activity and frequent contact with health professionals and reported significant beneficial effects on HbA1c, lipids, and blood pressure. All other trials either achieved a weight loss of < 5% and no benefit on HbA1c or CVD risk factors or found no differences between macronutrient interventions in weight or HbA1c.

LOOK AHEAD STUDY

The Look Ahead Study[67] enrolled 5145, aged 45-74 years, with BMI > 25 (> 27 if taking insulin) into a weight loss (with meal replacements if required) and exercise intervention. The Intensive lifestyle intervention produced an 8.6% weight loss at 1 year *vs* 0.7% in control group. Mean HbA1c dropped from 7.3% to 6.6%. At 4 years weight was still 5.3% lower compared with control and HbA1c-0.27% lower[68].

Although the study was ceased after 8 years because of lack of CVD differences compared with the control group[69] there were many benefits seen in the intervention in mood, quality of life and physical function[70]. It clearly showed that a weight loss of 10% or more could be achieved and maintained at 8 years in 27% of the intensive lifestyle group with 50% achieving more than 5% weight loss[71]. One of the reasons the trial failed to achieve its primary end point was because the support and education control group achieved a weight loss of 10% or more in 17% of the group with 5% or more weight loss achieved by 36%. The intervention led to reductions in hospitalizations (11%, $P = 0.004$), hospital days (15%, $P = 0.01$), and number of medications (6%, $P = 0.001$) compared with control participants who were invited to three sessions of diabetes support and education a year. No benefit was unfortunately seen in the 15% of the population with pre-existing CVD. There were fewer deaths in the intervention group (6.8% *vs* 7.8%) but this was not significant ($P = 0.15$)[72].

In secondary analyses of the full cohort[73] (both intervention and control groups), over a median 10.2 years of follow-up, individuals who lost at least 10% of their bodyweight in the first year of the study had a 21% lower risk of the primary outcome [death from CVD, MI, stroke or admission for angina (adjusted hazard ratio $P = 0.034$)] compared with individuals with stable weight or weight gain. In analyses treating the control group as the reference group, participants in the intensive lifestyle intervention group who lost at least 10% of their bodyweight had a 20% lower risk of the primary outcome $P = 0.039$.

ATKINS AND SOUTH BEACH DIETS

There is a small group of advocates for low carbohydrate Atkins style diets for clinical treatment in type 2 diabetes[74-76]. A 6-mo study from one group compared Atkins (LCKD) *vs* calorie-reduced low GI diet (LGID) in volunteers with a BMI 38, of whom 80% were women. There was a high dropout rate with 58.3% (49) participants completing. Body weight fell by 11.1 kg *vs* 6.9 kg ($P = 0.008$) and HbA1c was reduced by -1.5% *vs* -0.5% ($P = 0.03$). LDL was higher in the Atkins group by 4% which although small is of some theoretical concern[77]. There was no long term follow up which is important as Atkins adherence drops off dramatically after 6 mo. In a 48w study comparing an Atkins diet to a low fat diet plus orlistat in which 32% of the volunteers had type 2 diabetes ($n = 46$) weight loss was excellent in both groups at 8.65% to 9.5% with no differences between groups[78].

In an energy controlled low carbohydrate South Beach diet compared to a usual carbohydrate weight loss diet weight loss was the same as planned (9.8 and 10.1 kg) the overall HbA1c fall was the same but there was a greater effect in the low carbohydrate group at 6 mo if HbA1c was greater than 7.8% (2.6% *vs* 1.9%). Drug reductions were also greater in the South Beach group. At 12 mo the HbA1c difference had disappeared[34,79].

VLCD

Somewhat surprisingly the number of publications of

Table 2 Weight loss diets in people with type 2 diabetes

Type of diet	Type of summary document	Effect size	Long term data	Recommendation	Risk markers
Low glycemic index/low glycemic load	Canadian Trial of Carbohydrate in Diabetes[63] 12 mo study in 162 volunteers The HGI, LGI and LC diets contained 47% ± 1%, 52% ± 1% and 40% ± 1% energy carbohydrate; 30% ± 1%, 27% ± 1% and 40% ± 1% fat with GI 64 ± 0.4, 55 ± 0.4 and 59 ± 0.4	No difference between diets	None		
Low glycemic index	Canadian low glycemic index diet study[64] in 210 participants with type 2 diabetes on hypoglycemic medication	No effect on weight	None		HbA1c lower buy 0.32% on low glycemic index diet compared with high fibre diet
				No value in type 2 diabetes	
All randomised diets in type 2 diabetes of 12 mo or more duration	Eleven trials[65] were identified with 6754 participants were reviewed. Eight trials compared different diets while 3 compared diets to usual care. Only two study groups reported a weight loss of ⩾ 5%: A Mediterranean-style diet implemented in newly diagnosed adults with type 2 diabetes and an intensive lifestyle intervention implemented in the Look AHEAD (Action for Health in Diabetes) trial				
Conclusion				Mediterranean diet best	
Look ahead study	The Look Ahead Study[66] enrolled 5145, aged 45-74 yr, with BMI > 25 (> 27 if taking insulin) into a weight loss (with meal replacements if required) and exercise intervention	The Intensive lifestyle intervention produced an 8.6% weight loss at 1 yr *vs* 0.7% in control group	At 4 yr weight was still 5.3% lower compared with control. Weight loss of 10% or more at 8 yr in 27% of the intensive lifestyle group with 50% achieving more than 5% weight loss[70] support and education control group achieved a weight loss of 10% or more in 17% of the group with 5% or more weight loss achieved by 36%		Mean HbA1c dropped from 7.3% to 6.6% At 4 yr HbA1c-0.27% lower *Post hoc* analysis in the whole population (4834) over 10 yr[72] showed that those who lost at least 10% of their body weight in the first year had a 21% lower (HR 0.79, 95%CI: 0.64-0.98, *P* = 0.034) risk of primary outcome (death from CVD, MI, stroke, admission for angina), and a 24% reduced risk of the secondary outcome (primary plus CABG, carotid endarterectomy, stent, heart failure, PVD or total mortality) (adjusted HR 0.76, 95%CI: 0.63-0.91; *P* = 0.003)

Conclusion				Only non-surgical weight loss study with reduction in hard end points	
Atkins diet	A 6-mo study from one group of Atkins *vs* calorie-reduced low GI diet in volunteers with a BMI 38, of whom 80% were women[76]	Body weight fell by 11.1 kg *vs* 6.9 kg, P = 0.008 58.3% (49) participants completing			HbA1c was reduced by -1.5% *vs* -0.5% (P = 0.03) LDL was higher in the Atkins group by 4%
Atkins diet	48w study[77] comparing an Atkins diet to a low fat diet plus orlistat in which 32% of the volunteers had type 2 diabetes (n = 46)	Weight loss 8.65% to 9.5% with no differences between groups			
South Beach diet	80 volunteers completed a 12 mo very low carbohydrate diet *vs* an energy matched high carbohydrate diet[34,78]	9.8 and 10.1 kg at 12 mo			Hba1c changes different at 6 mo but not at 12.1% reduction
Conclusions				Low carbohydrate diets good in short term with intensive support	
VLCD	Meta-analysis of 5 studies of VLCD in volunteers with diabetes or no diabetes[80]	Weekly weight loss was similar in the two groups at 0.5 to 0.6 kg/wk. Weight losses of > 15%-20% were observed in these studies			
VLCD	Retrospective analysis of 355 patients with diabetes matched with nondiabetics	After 12 wk, there was significant weight loss within each group when compared with baseline (T2DM: 115.0 ± 24.4 kg *vs* 96.7 ± 21.4 kg, P < 0.0001; non-T2DM: 117.2 ± 25.8 kg *vs* 97.3 ± 22.2 kg, P < 0.0001)	No long term data available		
	Total cohort comprised 204 males: 506 females, age 54.0 ± 9.1; BMI 41.6 ± 8.1; weight 116.1 ± 25.1 kg[81]	At 12 wk, weight change (-18.3 ± 7.3 kg *vs* -19.9 ± 7.0 kg, P = 0.012) were significantly less in the T2DM group when compared with the non-T2DM group			
VLCD	40 individuals with type 2 diabetes and no control group	Weight loss of 10 kg at 1 yr after an 8 wk VLCD. Five year data from a comparison of self-selected VLCD (15) to modest caloric restriction (n = 15) showed better weight loss in the conventional diet 8.9 kg *vs* 4.8 kg[83] Early use of VLCD can cause remission of type 2 diabetes[84]	Long term data shows benefit	VLCD useful	
Conclusion				Although expensive VLCD has long term benefits	
Diet plus exercise	2 controlled studies adding aerobic or resistance exercise to significant weight loss over 12 to 16 wk[86,87]	No additional benefit of adding exercise on weight	No long term data		No additional benefit on HbA1c or any other markers
Conclusions				No added benefit	

CER: Continuous energy restriction; CHO: Carbohydrate; GI: Glycemic index; VLCD: Very low calorie diet.

the use of meal replacements and VLCD in diabetes is limited[80]. In a meta-analysis of 5 studies of VLCD in both people with and without diabetes there was no difference in achieved weight loss between these two groups. Weekly weight loss was similar in the two groups at 0.5 to 0.6 kg/wk. Weight losses of > 15%-20% were observed in these studies[81]. In a retrospective analysis[82] 355 participants with T2DM were matched for age, BMI and gender to participants without T2DM. The program included a daily intake of 550 kcal in addition to group support and behavior therapy provided by trained facilitators within a community-based setting. At 12 wk, weight change (-18.3 ± 7.3 kg *vs* -19.9 ± 7.0 kg, P = 0.012) was significantly less in the T2DM group when compared with the non-T2DM group. In a study of 40 individuals with type 2 diabetes and no control group Dhindsa *et al*[83] found a weight loss of 10 kg at 1 year after an 8 wk VLCD. Five year data from a comparison of self-selected VLCD (15) to modest caloric restriction (n = 15) showed better weight loss in the conventional diet 8.9 kg *vs* 4.8 kg[84]. Early use of VLCD can cause remission of type 2 diabetes[85].

Johansson *et al*[86] reviewed weight maintenance strategies and found that medication, meal replacements and high protein diets were helpful over a 5-18 mo period while exercise and supplements were not.

DIET PLUS EXERCISE

The final question we will examine in this review is whether exercise has additive benefits to weight loss. Wycherley *et al*[87,88] performed 2 studies adding aerobic or resistance exercise to significant weight loss over 12 to 16 wk and found no additional benefit of adding exercise on HbA1c or any other markers.

THE FINAL WORD FOR THIS REVIEW IS THE MICROBIOME

Rodent studies from Gordon *et al* taking germ-free mice and giving them a "fat" microbial population made them fat, while a lean microbial population keeps them lean[89,90]. Fat mice and lean mice[91] (and humans[92]) have different bacterial populations and the population changes as weight changes (Phyla: Firmicutes up and Bacteroidetes down with increased weight). An increase in calorie intake (from 2400 to 3400 kcal/d) in obese and lean human individuals promotes rapid changes in the gut microbiota (20% increase in *Firmicutes* and a corresponding decrease in *Bacteroidetes*) and this was associated with an increased energy harvest of approximately 150 kcal, the overfeeding in lean individuals being accompanied by a greater fractional decrease in stool energy loss[93].

Increasing dietary fat alters the microbiome, increases gut leakiness ans lipopolysaccharide absorption and enhances insulin resistance[94,95] while feeding oligofructans increase Bifido, reduce insulin resistance and inflammation[96]. Feeding flaxseed mucilage for 6w improved insulin resistance, altered 33 microbial species, lowered 8 including faecalibacterium. The species change could not be related to the change in insulin resistance[97]. Pedersen *et al*[98] fed a galacto-oligosaccharide mix (5.5 g/d) for 12 wk or placebo and demonstrated no changes in insulin sensitivity, glucose tolerance, gut leakiness, inflammatory markers or the microbiome. Changes in the bacterial family Veillonellaceae correlated inversely with changes in glucose response and IL-6 levels (r = -0.90, P = 0.042 for both) following prebiotic intake. Metformin may mediate some of its therapeutic effects through short-chain fatty acid production, while its intestinal adverse effects may be due to relative increase in abundance of Escherichia species. Controlling for metformin treatment, the gut microbiome shifts in T2D with a depletion of butyrate-producing taxa[99].

Weight loss induced by Roux on Y gastric bypass led to reduction of Firmicutes and Bacteroidetes and an increase of Proteobacteria and these species were related to BMI and CRP[100]. Faecalibacterium prausnitzii was directly correlated to fasting blood glucose. In an earlier study Faecalibacterium prausnitzii species was lower in subjects with diabetes and associated negatively with inflammatory markers at baseline and throughout the follow-up after surgery independently of changes in food intake[101].

CONCLUSION

Weight loss occurs with many different diets and there are no clear conclusions on the optimal diet apart from the diet which the individual can stick to long term, whatever the composition. Whether phenotyping (*e.g.*, degree of insulin resistance) or genotyping will help diet choice is not clear.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Neutral protamine hagedorn/regular insulin in the treatment of inpatient hyperglycemia: Comparison of 3 basal-bolus regimens

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Author contributions: Quintanilla-Flores DL and García-De la Cruz G were involved in conception and design of the study, acquisition of data, analysis and interpretarion of data; Tamez-Pérez HE outlined and coordinated the writing of the paper and added significant contributions to the text; González-González JG provided input in writing the paper and made significant contributions to the text.

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Abstract

AIM

To compare the safety and efficacy or 3 basal-bolus regimens of neutral protamine hagedorn (NPH)/regular insulin in the management of inpatient hyperglycemia.

METHODS

We randomized 105 patients with blood glucose levels

between 140 and 400 mg/dL to a basal-bolus regimen of NPH insulin given once (n = 30), twice (n = 40) or three times (n = 35) daily, in addition to pre-meal regular insulin. Major outcomes included were differences in glycemic control, frequency of hypoglycemia and total insulin dose.

RESULTS

NPH insulin given in a once-daily regimen was associated with better glycemic control (58.3%) compared to twice daily (42.4%) and three times daily (48.9) regimens (P = 0.031). The frequency of hypoglycemia was similar between the three groups (2.0%, 0.7% and 1.2%, P = 0.21). The mean insulin dose at discharge was 0.48 ± 0.14 U/kg in the once-daily group compared to 0.69 ± 0.28 in the twice-daily, and 0.65 ± 0.20 in the three times daily regimens (P < 0.001).

CONCLUSION

NPH insulin administered in a once-daily regimen resulted in improvement in glycemic control with similar rates of hypoglycemia compared to a twice-daily and a three times-daily regimen. Further studies are needed to evaluate whether this regimen could be implemented in all hospitalized patients with hyperglycemia.

Key words: Neutral protamine hagedorn insulin; Hospital hyperglycemia; Basal-bolus regimen; Type 2 diabetes mellitus; Inpatient care units

Core tip: In this parallel randomized clinical trial, we compared various insulin regimes. Administration of one-daily neutral protamine hagedorn (NPH) regimen improved glycemic control with similar rates compared to a twice-daily and a three times daily regimen. Furthermore, the use of NPH insulin in a once-daily regimen is associated with lower requirements as well as lower variability in the insulin dose during follow up.

Quintanilla-Flores DL, González-González JG, García-De la Cruz G, Tamez-Pérez HE. Neutral protamine hagedorn/regular insulin in the treatment of inpatient hyperglycemia: Comparison of 3 basal-bolus regimens. *World J Diabetes* 2017; 8(10): 455-463 Available from: URL: http://www.wjgnet.com/1948-9358/full/v8/i10/455.htm DOI: http://dx.doi.org/10.4239/wjd.v8.i10.455

INTRODUCTION

Hyperglycemia is a common finding in hospitalized patients with a prevalence of approximately 25%[1]. It can be secondary to undiagnosed diabetes, stress hyperglycemia pharmacological agents, glucocorticoids or poorly controlled diabetes. For every 2 patients hospitalized with a diagnosis of type 2 diabetes mellitus (DM2), there is one with previously undetected hyperglycemia[2]. In addition, about 90% of hospitalized patients with diabetes have hyperglycemia (> 200 mg/dL) and in 20% of these patients hyperglycemia persists for 3 or more days[3].

Poor glycemic control has been established as a risk factor for poor clinical outcome and mortality[2,4]. Glucose levels between 140-180 mg/dL are associated with a reduction in mortality, systemic infections, risk of multi-organ failure, bacetermia, critical illness polyneuropathy, inflammation and hospital stay[4-6]. Subcutaneous insulin, given as a daily basal-bolus, is the only agent that has proven efficacy and safety for glycemic control in general medical and surgical patients with hyperglycemia.

Despite its benefits, treatment of hyperglycemia still remains delayed. The fear of causing hypoglycemia[3] and the clinical inertia of no treatment remain the main barriers for initiating insulin. Physicians commonly use a sliding-scale regimen until stabilization of glucose levels[7]; however, a study by Umpierrez *et al*[8] found that a basal-bolus insulin algorithm was more effective than a sliding-scale regimen for glucose control.

The use of a basal-bolus regimen with both insulin analogs and a neutral protamine hagedorn (NPH)/regular insulin mix has been studied. Similar rates of glucose control and hypoglycemic events were found with both regimens making them suitable for the treatment of inpatient hyperglycemia[4,9,10]. Current guidelines do not specify whether the NPH dose of insulin should be administered in a once daily, twice daily or three times daily regimen during hospitalization. The twice daily regimen has been traditionally used in previous clinical trials as the standard regimen of reference, suggesting it to be the most physiologic form of administration. Accordingly, we conducted a prospective, randomized non-blinded study to compare the efficacy and safety of three basal-bolus regimens of NPH/regular insulin for the control of hyperglycemia in patients admitted to an internal medicine ward.

MATERIALS AND METHODS

Subjects

Subjects were men and women aged > 16 years, admitted to medical services with a persistent blood glucose level > 140 mg/dL and with an expected stay $\geqslant$ 48 h. Exclusion criteria included individuals with type 1 diabetes mellitus, parenteral nutrition, blood glucose levels $\geqslant$ 400 mg/dL at screening, diabetic ketoacidosis or nonketotic hyperosmolar syndrome, clinically relevant hepatic disease, glomerular filtration rate $\leqslant$ 30 mL/min, pregnancy, terminal disease, and/or inability to provide informed consent. Patients were eliminated when there was poor adherence to the administration of insulin or glucose measurements (defined as $\leqslant$ 70% of total insulin doses or glucose measurements), discharge or death within the first 48 h of enrollment or when glucocorticoids were given during follow up.

Study design

We developed a single center, open-label, randomized, parallel comparative study in the Internal Medicine Department, at the "Dr. José Eleuterio González" University Hospital from September 2013 to September 2015. It was conducted in accordance with the Declaration of Helsinki revised in 2008 and approved by the local ethical committees. All subjects provided informed consent. Participants were randomized using an online randomization generator available at http://www.randomization.com. A database including the sequential order of randomization was generated in an Excel file. Both the enrollment and follow-up of the included subjects was performed by the members of the research team in cooperation with the attending physicians. The protocol was registered in clinicalrials.gov (Trial registry number: NCT02758522).

Study protocol and treatment

All patients were managed by physicians of an internal medicine residency program. The primary care teams decided on the treatment for all other medical problems for which the patients were admitted. Oral antidiabetic drugs were suspended during hospitalization. HbA1c was measured during the first day of hospital stay. Post-discharge follow up was not included as part of this study.

Patients were randomized to receive NPH insulin either once-daily, twice-daily or three times-daily. The twice-daily regimen was also included as the reference regimen, since it has been traditionally used in previous trials when NPH/Regular insulin is administered in hospitalized patients. The starting dose was calculated according to body mass index (BMI): 0.3 U/kg for BMI < 18 kg/m^2, 0.4 U/kg for BMI 18-24.9 kg/m^2, 0.5 U/kg for BMI 25-29.9 kg/m^2 and 0.6 U/kg for BMI $\geqslant$ 30 kg/m^2. The resulting dose was fractioned to be given 60% as basal insulin (NPH) and 40% as prandial (regular) insulin. NPH insulin once-daily was administered subcutaneously before breakfast; in the twice-daily regimen it was given before breakfast and before dinner; and in the three times daily regimen it was administered before each meal. Regular insulin was given in three equally divided doses before each meal. A sliding-scale regimen of supplemental regular insulin was given in addition to the scheduled pre-meal insulin when blood glucose levels were $\geqslant$ 140 mg/dL. When the patient was not able to eat, the dose of regular insulin was held until meals were resumed. Furthermore, when glucose values between 70 mg/dL and 100 mg/dL were detected before meals, the corresponding dose of insulin was suspended in order to prevent hypoglycemia.

Hypoglycemia was defined as a glucose level < 70 mg/dL. Severe hypoglycemia was defined as a glucose level < 40 mg/dL or the need of assistance. All blood glucose values less than 70 mg/dL were treated with 20 g oral carbohydrate (fruit or juice) or 25 g of intravenous glucose depending on the neurologic state. The dose of total daily insulin was reduced by 20% when an episode of hypoglycemia was reported.

Blood glucose was determined four times a day: Before each meal and at bedtime using a glucose meter. The insulin dose was adjusted daily according to glucose values: If blood glucose was not in the target range of fasting glucose $\leqslant$ 140 mg/dL and random glucose was $\leqslant$ 180 mg/dL (nonfasting glucose measured at any time during the day), the total insulin dose was increased by 20%, fractioned in 60% NPH and 40% rapid insulin.

Outcome measures

The primary outcome was to determine the differences in glycemic control between the treatment groups. Glycemic control was defined as the proportion of patients that achieved fasting glucose between 70-140 mg/dL and random glucose levels of < 180 mg/dL during the whole hospital stay. Mean overall, fasting and random, glucoses were also used to assess differences in glycemic control between the three regimens. They were established as the average of daily repeated measurements taken each day during hospitalization. Secondary outcomes included differences in the percentage of glucose levels in the hypoglycemic range (overall and severe hypoglycemia), and the total insulin dose required during follow up and at discharge to achieve glycemic control and differences in mortality and hospital stay.

Statistical analysis

Based on previous data about glycemic control in hospitalized patients, we calculated that 93 subjects (31 per group) had the power to provide an 80% chance of detecting, with an α error rate of 5%, a difference greater than 30% in glycemic control between the 3 regimens. Data were analyzed using SPSS version 19.0 software package. For the continuous variables, differences were examined by ANOVA or Kruskal Wallis as needed. The χ^2 test was used for categorical data. $P < 0.05$ was considered significant.

RESULTS

A total of 105 patients were finally included for analysis, 85 of them with known type 2 diabetes mellitus. Figure 1 shows the enrollment of the patients. No between-treatment differences were apparent at baseline, except that patients in the once-daily regimen had a shorter duration of diabetes (P = 0.01) and were less prone to insulin use before hospitalization (P = 0.01) (Table 1). Metformin and glibenclamide were the only oral anti-diabetic drugs used by the patients prior hospitalization. These drugs were drugs were suspended during hospitalization. Over 19% subjects had an unrecognized history of diabetes mellitus, and more than half had received prior therapy with insulin before hospitalization. The most common diagnoses on admission were coronary artery disease, infections and neoplastic disorders. Pneumonia was the most common

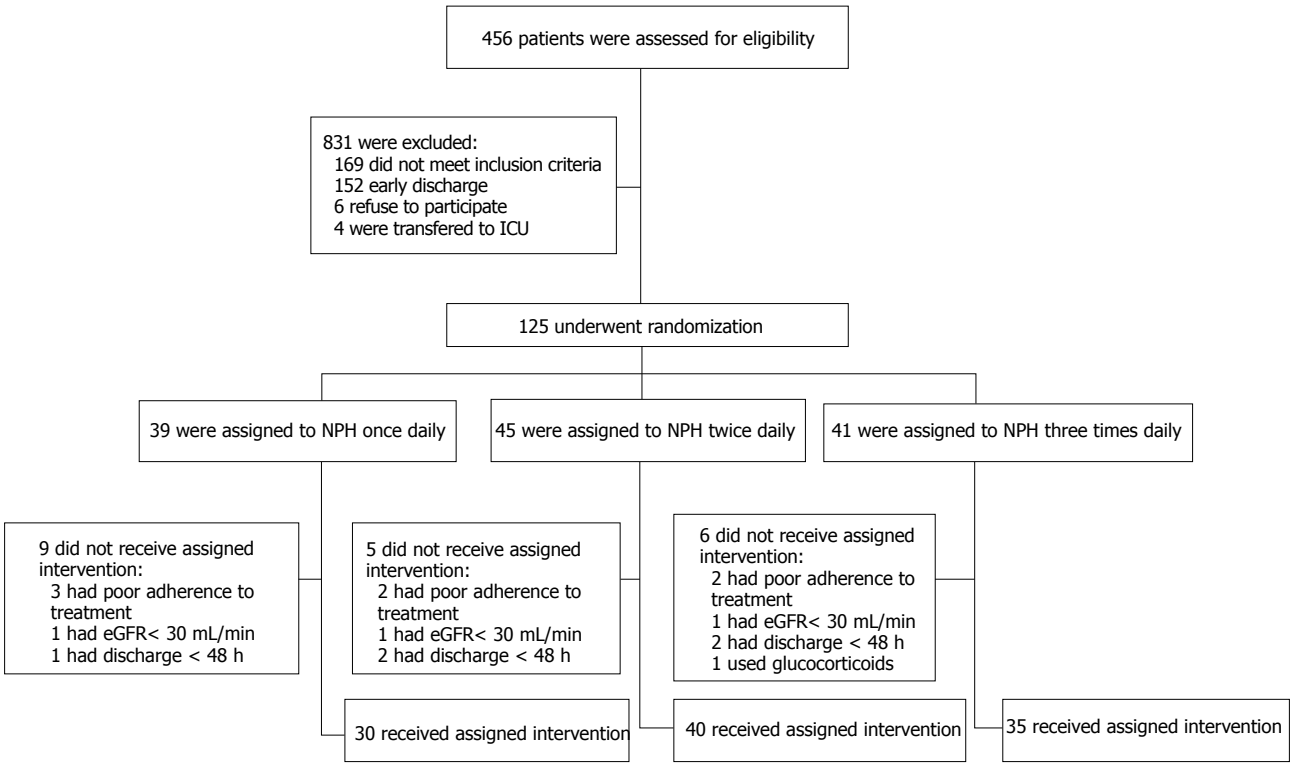

Figure 1 Enrollment and randomization of patients.

Table 1 Baseline clinical characteristics

	NPH × 1	NPH × 2	NPH × 3	*P*
n	30	40	35	
Age (yr, X ± DS)	60 ± 15	58 ± 15	54 ± 14	0.39
Gender (% female)	12 (40)	20 (50)	22 (63)	0.18
Unknown history of T2DM, *n* (%)	12 (40.0)	4 (10.0)	4 (11.4)	0.01
Duration of T2DM (yr), med (min-max)	5 (0-30)	15 (0-30)	10 (0-25)	0.01
Prior T2DM therapy, *n* (%)				0.02
None	17 (56.7)	7 (17.5)	9 (25.7)	
Oral antidiabetics	9 (30.0)	20 (50.0)	15 (42.9)	
Insulin	4 (13.3)	21 (52.5)	15 (42.9)	
Insulin + oral antidiabetics	-	8 (20.0)	4 (11.4)	
Charlson score, med (min-max)	3 (1-9)	3 (1-5)	3 (1-7)	0.14
Hospitalization diagnosis, *n* (%)				
Coronary artery disease	7 (23.3)	13 (32.5)	11 (31.4)	0.69
Infectious disease	5 (16.7)	13 (32.5)	9 (25.7)	0.35
Neoplasm	7 (23.3)	3 (7.5)	2 (5.7)	0.051
Dysrhythmias	4 (13.3)	1 (2.5)	2 (5.7)	0.23
Gastrointestinal hemorrhage	4 (13.3)	1 (2.5)	3 (8.6)	0.24
Pancreatitis	2 (6.7)	1 (2.5)	1 (2.9)	0.68
Stroke	-	2 (5.0)	1 (2.9)	0.78
Other	1 (3.3)	6 (15.0)	6 (15.0)	0.88
Hypertension, *n* (%)	8 (26.7)	12 (31.6)	15 (42.9)	0.33
Body mass index (kg/m^2), X ± DS	26.4 ± 5.2	27.5 ± 5.6	27.5 ± 5.3	0.65
HbA1c (%), X ± DS	9.5 ± 2.4	10.2 ± 2.4	10.4 ± 2.8	0.45
HbA1c (mmol/mol)	80 ± 26	88 ± 26	90 ± 30	
Admission blood glucose (mg/dL), X ± DS	272 ± 84	308 ± 62	306 ± 70	0.08
Glomerular filtration rate[1] (mL/min), X ± DS	77.3 ± 32.9	86.9 ± 30.1	92.4 ± 23.4	0.13
Treatment follow-up (d), med (min-max)	6 (2-14)	6 (2-14)	7 (2-14)	0.41
Hospital stay (d), med (min-max)	8 (4-31)	8 (2-28)	10 (4-36)	0.39

[1]Calculated with Chronic Kidney Disease Epidemiology Collaboration. T2DM: Type 2 diabetes mellitus; HbA1c: Glycosylated hemoglobin.

cause of infection, followed by urinary tract infections and diarrhea. None of the subjects with sepsis were included.

The median duration of treatment was 6 (2-14) d, and the median hospital stay was 8 (2-36) d. No deaths were

Table 2 Glycemic control and insulin dose[1]

	NPH × 1, *n* = 30	NPH × 2, *n* = 40	NPH × 3, *n* = 35	*P*
Mean glucose (mg/dL)	160.3 ± 36.4	190.4 ± 48.0	178.7 ± 44.2	0.02
Fasting glucose (mg/dL)	149.2 ± 36.5	175.9 ± 54.6	169.5 ± 43.2	0.054
Random glucose (mg/dL)	164.4 ± 38.2	198.9 ± 53.2	181.0 ± 47.8	0.013
Glycemic control (%)	58.3 ± 25.3	42.4 ± 24.3	48.9 ± 24.1	0.031
Fasting glucose (%)	47.0 ± 35.0	34.0 ± 30.8	42.5 ± 32.3	0.253
Random glucose (%)	62.8 ± 25.9	45.5 ± 25.2	52.8 ± 26.6	0.024
50% daily glucoses within target range (%)	53.0 ± 29.4	43.8 ± 29.5	48.1 ± 30.6	0.455
Time to achieve 50% of daily glucoses within target range (h)	48.9 ± 27.8	61.2 ± 33.9	59.6 ± 47.0	0.438
75% daily glucoses within target range (%)	27.4 ± 26.5	14.3 ± 21.1	21.8 ± 25.5	0.069
Time to achieve 75% of daily glucoses within target range (h)	76.8 ± 48.4	84.8 ± 57.3	99.8 ± 85.1	0.904
Insulin dose (UI/kg)				
Basal	0.44 ± 0.13	0.51 ± 0.18	0.52 ± 0.15	0.1
At discharge	0.48 ± 0.14	0.69 ± 0.28	0.65 ± 0.20	< 0.001
Δ Insulin dose	0.04 ± 0.10	0.19 ± 0.22	0.13 ± 0.18	0.004

[1]Data are expressed as X ± SD.

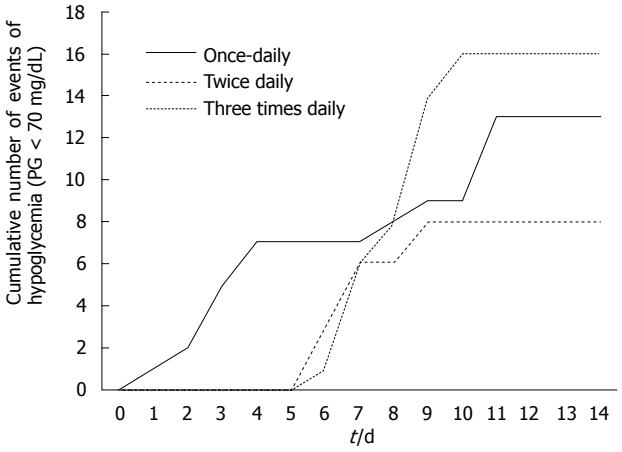

Figure 2 Cumulative number of hypoglycemia events. Pearson χ^2 (P = 0.004).

reported among the study subjects. Diabetes related chronic complications were not evaluated in this study.

Glycemic response and insulin dose

Mean baseline glucose levels were similar between the three groups. Mean glucose levels during follow up were 160, 190 and 179 mg/dL for the once-daily, twice-daily and three times-daily regimens, respectively (P = 0.02). The percentage of patients within the target range of glycemic control were 58% in patients treated with the once-daily regimen, 42% in the twice-daily regimen and 49% in the three times-daily regimen (P = 0.03). In the *post-hoc* analysis patients treated with the once-daily regimen had greater improvement in glycemic control than those treated with the twice-daily regimen (P = 0.03), maintaining significant differences only in random glucose samples (P = 0.02). There was no significant difference between the subjects in the once-daily regimen and the three times-daily regimen. Nearly half of the patients achieved had least 50% of the glucose measures of the day within the target ranges (P = 0.39), and about one quarter achieved 75% within the target ranges (P = 0.09) (Table 2).

The once-daily regimen provided glycemic control when the duration of diabetes was < 10 years, the patient received treatment with insulin before hospitalization, the HbA1c was > 9% (75 mmol/mol), there was an absence of infection and the BMI was ⩾ 25 kg/m^2 (Table 3).

Mean total insulin daily doses were significantly higher in both the three times-daily and the twice-daily regimens compared with that in the once-daily regimen (P < 0.001). Furthermore the once-daily regimen was associated with less variability in insulin dose during the entire study, as shown in the Δ of insulin dose (P = 0.004) (Table 2).

Rate of hypoglycemia

Figure 2 shows the cumulative incidence of hypoglycemic events. Fewer events occurred with the twice-daily regimen, followed by the once-daily regimen, and the three times-daily regimen (P = 0.004). Expressed as rate of hypoglycemia (proportion of events/total glucoses), the differences did not reach statistical significance. A total of 492 glucose readings were performed in the once-daily regimen; of these 13 (2.0%) were < 70 mg/dL. Of the 754 glucose readings in the twice-daily regimen 8 (0.7%) were < 70 mg/dL. Finally, of the 745 glucose readings of the three times-daily regimen 16 (1.2%) were < 70 mg/dL (P = 0.21). Only one episode of severe hypoglycemia was documented in the twice-daily regimen.

A higher proportion of patients in the three times-daily regimen experienced hypoglycemia before dinner (P = 0.04). The insulin dose of presentation of an event of hypoglycemia was significantly lower in the once-daily regimen (0.38 ± 0.13 U/kg) compared to the twice-daily (0.67 ± 0.17 U/kg) and the three times-daily [0.94 ± 0.48 (U/kg)] regimens (P < 0.001) (Table 4). When adjusting the rate of hypoglycemia according to different variables, the once-daily regimen proved to be associated with higher rates when HbA1c < 9% (75 mmol/mol) (rate 4.3%) compared to the twice daily

Table 3 Glycemic control among subgroups

	NPH × 1, *n* = 30, (%)	NPH × 2, *n* = 40, (%)	NPH × 3, *n* = 35, (%)	*P*
DM ⩽ 10 yr				
Overall	62.1 ± 24.8	47.3 ± 25.6	50.4 ± 23.4	0.17
Fasting glucose	53.7 ± 31.9	35.2 ± 30.5	42.1 ± 32.0	0.03
Random glucose	65.9 ± 24.9	51.1 ± 27.2	55.8 ± 28.2	0.25
Pre-hospital insulin				
Overall	77.5 ± 12.4	37.6 ± 23.9	37.7 ± 26.1	0.012
Fasting glucose	39.5 ± 35.5	29.7 ± 27.5	24.1 ± 25.1	0.59
Random glucose	91.8 ± 7.5	41.0 ± 26.1	46.0 ± 31.2	0.01
Baseline glucose > 300 mg/dL				
Overall	52.9 ± 24.5	37.7 ± 26.9	40.8 ± 20.0	0.36
Fasting glucose	38.1 ± 35.6	33.6 ± 34.8	36.0 ± 26.7	0.94
Random glucose	57.4 ± 22.4	38.7 ± 26.1	42.7 ± 22.5	0.21
HbA1c > 9% (75 mmol/mol)				
Overall	55.2 ± 24.0	33.7 ± 22.6	45.8 ± 28.1	0.06
Fasting glucose	43.0 ± 33.9	25.5 ± 27.3	40.4 ± 31.0	0.18
Random glucose	60.0 ± 22.3	36.6 ± 23.1	48.2 ± 28.3	0.04
Absence of infectious disease				
Overall	61.0 ± 24.1	39.8 ± 25.1	50.9 ± 25.6	0.01
Fasting glucose	50.8 ± 34.0	34.2 ± 32.8	44.1 ± 35.8	0.22
Random glucose	65.4 ± 25.4	41.8 ± 25.3	54.3 ± 26.5	0.01
Glomerular filtration rate < 60 mL/min				
Overall	62.8 ± 25.3	42.0 ± 29.7	45.2 ± 16.7	0.20
Fasting glucose	40.0 ± 34.7	35.1 ± 32.8	31.2 ± 20.3	0.87
Random glucose	71.9 ± 27.3	44.4 ± 30.4	55.4 ± 29.5	0.14
Body mass index, dex ± 29.52				
Overall	63.4 ± 22.8	44.1 ± 25.2	48.0 ± 23.3	0.03
Fasting glucose	47.1 ± 35.0	39.9 ± 34.0	42.1 ± 30.4	0.78
Random glucose	69.9 ± 23.1	45.6 ± 25.1	50.8 ± 23.9	0.01

Proportion of patients that achieved glycemic targets during the whole follow up. Data are expressed as X ± SD. DM: Diabetes mellitus; HbA1c: Glycosylated hemoglobin.

Table 4 Rate of hypoglycemia among the study groups during the hospitalization

	NPH × 1, *n* = 30	NPH × 2, *n* = 40	NPH × 3, *n* = 35	*P*
Hypoglycemic events (*n*)	13	8	16	
Severe hypoglycemia	–	1	–	0.45
Rate of hypoglycemia (%), (X ± SD)[1]	2.0 ± 3.8	0.7 ± 2.3	1.2 ± 3.1	0.21
Time to the first episode (d), (X ± SD)	6.2 ± 4.0	7.1 ± 1.2	8.2 ± 1.2	0.14
Insulin dose at event (IU/kg), (X ± SD)	0.38 ± 0.13	0.67 ± 0.17	0.94 ± 0.48	< 0.001
Time of presentation, *n* (%)				
Before breakfast	5 (38.5)	2 (25.0)	1 (6.2)	0.11
Before supper	3 (23.1)	3 (37.5)	2 (12.5)	0.37
Before dinner	2 (15.4)	–	7 (43.8)	0.04
Bedtime	3 (23.1)	3 (37.5)	6 (37.5)	0.43

[1]Data are expressed as proportion of events/total glucoses.

regimen (rate 1.1%) and the three times daily regimen (rate 0%) (P = 0.04).

DISCUSSION

NPH insulin administered in a once-daily regimen resulted in improvement in glycemic control with similar rates of hypoglycemia compared to a twice-daily and a three times-daily regimen. This superiority is of particular importance when the duration of diabetes is less than 10 years, HbA1c > 9% (75 mmol/mol), there is pre-hospital insulin use, an absence of infection during hospitalization and the patient has a BMI ⩾ 25 kg/m^2. Furthermore, the use of NPH insulin in a once-daily regimen is associated with lower insulin requirements and lower variability in the insulin dose during follow up.

According to previous studies[4,9,10], glycemic control with levels < 140 mg/dL can be achieved in up to 48%-74% of patients with rates of hypoglycemia of 2%-3.3% when scheduled NPH/regular insulin in a twice-daily protocol is used in non-critically ill patients. We found differences in glucose levels and lower rates of hypoglycemia when a twice-daily regimen was implemented. This could be explained by differences in the target glucose values in previous studies as well as the variability in the basal characteristics of our patients,

who had a longer duration of diabetes, higher HbA1c levels and a higher proportion of individuals using insulin prior to randomization. Furthermore, our population included only Hispanic subjects, which according to Bueno *et al*[10] tend to be significantly leaner, have worse glycemic control and higher HbA1c levels on admission as well as more hypoglycemic events compared to United States population.

In the ambulatory setting, the addition of a single bedtime injection of NPH insulin in those patients who remain poorly controlled with oral agents has been explored[11]. Extrapolated to the hospital setting, this is the first prospective randomized study that evaluates the efficacy of NPH insulin given in a once-daily regimen to inpatients with hyperglycemia. Of note is the observation that compared to the other two study groups, NPH insulin given in a once-daily regimen was associated with a lower dose of total insulin at the end of the study as well as with less variability in the insulin dose during the study period. Despite these differences in total insulin dose, this regimen was related to better glycemic control in selected patients as well as similar rates of hypoglycemia. This measure should be recommended especially when the duration of diabetes is < 10 years, the patients have been treated with insulin prior to hospitalization, HbA1c is > 9% (75 mmol/mol), an absence of infection, and the patient's BMI $\geqslant$ 25 kg/m^2.

Compared to insulin analogs, variability in the serum levels of NPH insulin, secondary to intermediate duration of action and a peak activity at 4-6 h after injection, have questioned its safety and efficacy in the treatment of hyperglycemia. NPH insulin has proved similar rates of glycemic control with a tendency to higher risk of hypoglycemia and greater glycemic variability when it is compared with glargine or detemir[4,11]. Some other studies have concluded similar rates of glycemic control and hypoglycemia[9]. In an attempt to equalize the effect of insulin analogs in terms of glycemic variability, we tried to split the total dose of NPH insulin into 3 equal doses administered during the day. We hypothesized that by splitting the total dose of NPH insulin, we could achieve a flat curve of serum NPH insulin levels similar to that observed with insulin analogs. On the contrary, we found higher rates of a cumulative number of hypoglycemia events and higher doses of insulin required to achieve similar rates of glycemic control. It seems that this measure should not be used as a first-line option in the management of inpatient hyperglycemia. It might be useful when higher doses of total insulin are required during the follow-up of patients treated with a once or twice daily regimen.

Controversy exists whether insulin analogs, such as glargine and detemir, are associated with better glycemic control and a lower risk of hypoglycemia compared to NPH insulin in the management of hospitalized hyperglycemia in the non-crically ill. Yeldandi *et al*[4] showed similar rates of glycemic control with a lower risk of hypoglycemia when insulin glargine was used compared to NPH insulin in a basal/bolus scheme. In the DEAN trial, similar improvements in glycemic control with no differences in hypoglycemia events were found with the use detemir once daily and aspart before meals compared to NPH/regular insulin in a twice daily regimen[9]. Bueno *et al*[10] showed similarly significant improvement in glycemic control without increasing the prevalence of overall hypoglycemia, with higher prevalence of severe hypoglycemia when twice daily NPH/regular insulin was used compared to once daily glargine and glulisine before meals (0.83% *vs* 0.25%, P = 0.01)[10]. In institutions with low- and middle-income resources, such as ours, access to insulin analogs is barely possible. It seems that the benefits of optimal glycemic control outweigh the slightly increased risk of severe hypoglycemia, which of note does not exceed 1% in overall prevalence. We consider that the implementation of protocols of glycemic control that include the use of NPH insulin in the basal regimen are still needed to reduce the complications of severe hyperglycemia and hypoglycemia in hospitalized patients.

There are several limitations in our study to consider: (1) we did not assess the daily oral caloric intake of our patients and the stratification of risk factors of hypoglycemia. Higher risk of hypoglycemia has been observed among subjects with variability in their caloric intake, comorbidities such as liver disease and renal disease, sepsis, malnutrition and drugs such as quinolones and β-agonists[12]; (2) our study was powered to evaluate differences in glycemic control and risk of hypoglycemia instead of mortality and clinical outcomes. Despite the fact that 16% of the randomized patients were lost during follow up, the minimum of 93 subjects to maintain the statistical power of our study was accomplished. In addition, only patients who completed the study were included for the analysis. We believe that in spite of this limitation, our findings provide reliable information to draw conclusions; (3) we included patients with a longer duration of diabetes, higher HbA1c levels on admission and a greater proportion of patients on insulin before hospitalization compared to previous studies. This could underestimate the rates of glycemic control in our patients compared to that of previous studies which included subjects with lower risk of severe hyperglycemia as shown by Pasquel *et al*[13] who proved that patients with higher HbA1c levels have lower odds of having optimal glucose control among hospitalized patients; (4) as it is shown in Table 2, patients in the once-daily regimen had a shorter duration of diabetes and were less prone to insulin use before hospitalization. Additionally, the proportion of patients with unknown history of diabetes was substantially greater in this group as compared to others, the rate of hypoglycemia tended to be higher and the meantime insulin dose at the event was lower, indicating probable greater insulin sensitivity. These features could explain the better glycemic response and lower insulin dose in once-daily regimen group instead of the once-daily regimen itself; (5) we are aware that the comparison of repetitive measurements

could be a better strategy for statistical analysis, however we decided to use average glucose levels since this is the way it has been presented in previous studies that compare different schemes of treatment of inpatient hyperglycemia; and (6) even though subjects were treated with the insulin regimen during the whole hospitalization, the median duration of days for follow up in our study was 6 (2-14) d. This period of maximum 14 d of follow up permitted an adequate titration of insulin dose with achievement of glycemic target in all patients and avoided bias linked to long hospital stay related complications.

Conclusion

In summary, NPH insulin administered in a once-daily regimen resulted in improvement in glycemic control with similar rates of hypoglycemia compared to a twice-daily and a three times-daily regimen. This superiority is of particular importance when the duration of diabetes is less than 10 years, HbA1c is > 9% (75 mmol/mol), there is pre-hospital insulin use, an absence of infection during hospitalization and the patient's BMI $\geqslant$ 25 kg/m^2. Furthermore, the use of NPH insulin in a once-daily regimen is associated with lower requirements as well as lower variability in the insulin dose during follow up. Whether this superiority in glycemic control and insulin dose was related to greater insulin sensitivity among the study subjects in the once-daily regimen needs to be reassessed in further studies. NPH insulin in a three times-daily regimen might not be recommended as a first-line option, because it is associated with a higher cumulative incidence of hypoglycemia and higher insulin doses in spite of an equivalent glycemic control. In this parallel randomized clinical trial, we compared various insulin regimes. Administration of once-daily NPH regimen improved glycemic control with similar rates compared to a twice-daily and a three times daily regimen. Furthermore, the use of NPH insulin in a once-daily regimen is associated with lower requirements as well as lower variability in the insulin dose during follow up.

Despite its limitations, our findings could be useful for changing algorithms for the treatment of inpatient hyperglycemia in addition to current health policies. Further studies are needed to estimate whether NPH insulin in a once-daily regimen can be incorporated as an option in certain populations among the hospitalized patients.

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COMMENTS

Background

Poor glycemic control among hospitalized patients has been established as a risk factor for poor clinical outcome and mortality. The use of a basal-bolus regimen with both insulin analogs and a neutral protamine hagedorn (NPH)/regular insulin has proven efficacy and safety for glycemic control in general medical and surgical patients with hyperglycemia.

Research frontiers

In institutions with low- and middle-income resources, access to insulin analogs is barely possible. The implementation of protocols of glycemic control that include the use of NPH insulin in the basal regimen are still needed to reduce the complications of severe hyperglycemia and hypoglycemia in hospitalized patients.

Innovations and breakthroughs

In this study the authors showed that NPH insulin administered in a once-daily regimen results in improvement in glycemic control with similar rates of hypoglycemia compared to a twice-daily and a three times-daily regimen. Furthermore, it is associated with lower requirements as well as lower variability in the insulin dose during follow up.

Applications

This study provides evidence of an alternative regimen of basal/bolus insulin among the hospitalized patients with diabetes.

Terminology

Glycemic control was defined as the achievement of fasting glucose between 70-140 mg/dL and random glucose levels of < 180 mg/dL. Hypoglycemia was defined as a glucose level < 70 mg/dL. Severe hypoglycemia was defined as a glucose level < 40 mg/dL or the need of assistance.

Peer-review

This is an overall good quality article.

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REVIEW

Bariatric surgery and long-term nutritional issues

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Abstract

Bariatric surgery is recognized as a highly effective therapy for obesity since it accomplishes sustained weight loss, reduction of obesity-related comorbidities and mortality, and improvement of quality of life. Overall, bariatric surgery is associated with a 42% reduction of the cardiovascular risk and 30% reduction of all-cause mortality. This review focuses on some nutritional consequences that can occur in bariatric patients that could potentially hinder the clinical benefits of this therapeutic option. All bariatric procedures, to variable degrees, alter the anatomy and physiology of the gastrointestinal tract; this alteration makes these patients more susceptible to developing nutritional complications, namely, deficiencies of macro- and micro-nutrients, which could lead to disabling diseases such as anemia, osteoporosis, protein malnutrition. Of note is the evidence that most obese patients present a number of nutritional deficits already prior to surgery, the most important being vitamin D and iron deficiencies. This finding prompts the need for a complete nutritional assessment and, eventually, an adequate correction of pre-existing deficits before surgery. Another critical issue that follows bariatric surgery is post-operative weight regain, which is commonly associated with the relapse of obesity-related co-morbidities. Nu-tritional complications associated with bariatric surgery can be prevented by life-long nutritional monitoring with the administration of multi-vitamins and mineral supplements according to the patient's needs.

Key words: Bariatric surgery; Nutrient deficiency; Roux-en-Y gastric bypass; Sleeve gastrectomy; Pre-operative

deficit; Weight regain

Core tip: Bariatric surgery is increasingly and successfully applied for the treatment of morbid obesity. In spite of multiple clinical benefits, *i.e.*, durable weight loss and improvement/reversal of many comorbidities, a number of nutritional complications can develop especially in the long term, which could cause serious detriment to patients' health. We examine some important clinical conditions that are caused by the deficit of vitamins and micronutrients, such as anemia, osteoporosis, and malnutrition. We also discuss the importance of careful pre-operative assessments and the correction of pre-existing nutritional deficiencies, and present the current recommendations for an appropriate biochemical and nutritional monitoring in the long term.

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INTRODUCTION

Obesity has become an important public health priority because it increases the risk of comorbid conditions, including diabetes, cardiovascular disease and several types of cancers. In addition, it affects life quality and expectancy[1]. The impact of obesity on life expectancy has been well documented. Worldwide, over 2.5 million deaths annually can be attributed to obesity. Of particular concern is the growing economic burden that the care of obesity and its complications imposes on society and the health care system[2].

The increasing prevalence of obesity and comorbid conditions worldwide prompts for effective strategies for both treatment and prevention[1]. The treatment of obesity includes lifestyle changes (dietary restrictions and increased physical activity), the use of medications, and in some cases, surgery. Lifestyle changes can cause a 2%-6% weight loss; however, after 1-5 years, almost 90% of the patients have returned to their original weight or might even gain some weight. Drug treatment in general leads to a 5%-15% weight loss and should be considered only as an adjunct to lifestyle changes. Unfortunately, with respect to lifestyle intervention, medical treatment rarely yields satisfactory results in the long term[1,3].

Bariatric surgery has proven to achieve greater weight loss than non-surgical management and, most importantly, has proven to maintain it in the long term[4]. Thus, in patients with morbid obesity, *i.e.*, a body mass index of $\geqslant$ 40 or $\geqslant$ 35 kg/m^2 with co-morbidities, bariatric surgery is presently considered to be the only effective therapy for obesity. Extensive data demonstrate that surgery can improve or even reverse many comorbidities, such as type 2 diabetes, hypertension, obstructive sleep apnea and steatohepatitis[5-7]. With regard to type 2 diabetes, observational and randomized controlled trials with a follow-up duration of up to 5 years have established the superiority of bariatric surgery over medical therapy at achieving remission of the disease and improvement of the overall cardiovascular risk profile[8-10]. One of the longest weight-loss studies - the Swedish Obese Subjects - evaluated the long-term effects of different bariatric procedures and demonstrated significant reductions in cardiovascular and cancer-related mortality as well as significant improvement in the quality of life[11-13].

In spite of multiple clinical benefits, a number of surgical and gastrointestinal complications can occur following bariatric procedures, although the diffusion of the laparoscopic approach and the expansion of centers of excellence have greatly reduced the rate of post-operative mortality and adverse events[14]. The mean mortality rate is 0.3% for all procedures, which is comparable to those for hip replacement (0.3%) or laparoscopic cholecystectomy (0.3%-0.6%). Indeed, even lower mortality rates (0.04-0.13) are achieved in high-volume obesity centers[14]. Among the possible complications, nutritional deficiencies deserve careful consideration. They can develop as a consequence of reduced intake and/or malabsorption of nutrients and are more commonly seen after malabsorptive or mixed procedures in comparison to the restrictive procedures. Other causal factors include pre-operative deficiencies, post-surgery food intolerance, changes in taste and eating patterns and non-adherence to dietary and supplement recommendations. Nutritional deficiencies can present with a wide range of clinical manifestations, depending on the specific nutrients/micronutrients that are involved, the severity, and the duration of the deficiency states. Because they could cause serious detriment to patients' everyday lives and, in some instances, could result in life-threatening complications, a nutritional screening both before and after surgery is strongly recommended.

This review focuses on the main nutritional issues related to bariatric procedures by examining some important clinical conditions that are caused by the deficit of vitamins and micronutrients, such as anemia, osteoporosis, neurologic disorders, and malnutrition. We will also discuss the importance of careful pre-operative assessments and the correction of pre-existing nutritional deficiencies, which are quite common in obese patients. Last, recommendations for the prevention and treatment of nutritional deficiencies after bariatric surgery are presented.

CONVENTIONAL BARIATRIC SURGICAL PROCEDURES

Surgical procedures are generally classified into restrictive procedures, in which the stomach's capacity

is greatly reduced, malabsorptive procedures, in which malabsorption is the primary driver of the weight loss, or a combination of restrictive and malabsorptive elements (Figure 1). However, over the past few years, it has become clear that weight loss is not only due to reduced food intake and/or absorption induced by modification of gastrointestinal anatomy but also a consequence of changes in neural and gut hormonal signals that regulate hunger and satiety, gut microbiota, intestinal nutrient sensing, food preferences, and possibly energy expenditure[15]. These so-called weight-independent mechanisms contribute to a variable extent to weight loss and metabolic improvement, depending on the type of surgical technique.

***Laparoscopic adjustable gastric banding* (AGB)**

An adjustable silicone band is placed around the upper stomach, a few centimeters below the cardia, creating a 15 to 30 mL gastric pouch. The diameter of the outlet can be changed by injection of or removal of saline through a portal placed in the subcutaneous tissue that is connected to the band.

***Roux-en-Y gastric bypass* (RYGB)**

A small, vertically oriented gastric pouch is created, which remains attached to the esophagus at one end and, at the other end, is connected to a small section of the small intestine, thus bypassing the remaining stomach and the initial loop of the small intestine.

***Sleeve gastrectomy* (SG)**

The operation involves division of the stomach vertically, which reduces its size by 75%. The pyloric valve at the bottom of the stomach is preserved such that the stomach function and digestion remain unaltered. The procedure is not reversible and might be a first stage procedure to a RYGB or duodenal switch.

***Biliopancreatic diversion* (BPD)**

The operation consists of a distal horizontal gastrectomy that leaves a 200-250 mL of upper stomach. This remnant stomach is anastomosed to the distal 250 cm of small intestine (alimentary limb). The excluded small intestine (carrying bile and pancreatic secretion), called the biliopancreatic limb, is connected to the small bowel 50 cm proximal to the ileocecal valve. The 50-cm common limb is the only segment where digestive secretions and nutrients mix, which causes a marked malabsorption, especially for fat and protein.

A recent survey by the International Federation for the Surgery of Obesity showed that RYGB and SG account for the large majority of bariatric procedures (45% and 37%, respectively). The use of ABG has drastically fallen during the last decade and currently accounts for 10% of all procedures. BPD and its duodenal switch (BPD/DS)variant, which are truly malabsorptive procedures, are rarely used (< 2%) to date given the high risk of nutritional complications[16].

NUTRITIONAL ISSUES AFTER BARIATRIC SURGERY

Anemia

According to a recent report from the American Society of Hematology, people who have undergone bariatric procedures show the highest risk for anemia, with 33%-49% of operated patients presenting anemia within 2 years after surgery[17]. As expected, the average prevalence of anemia is lower following LSG (17%) and reaches 45%-50% after RYGB and BPD. It should be noted that, as underlined for other nutrient deficiencies, up to 10%-12% of obese patients already have anemia before surgery[18]; thus, baseline screening for anemia is recommended in all patients who are scheduled for bariatric procedures.

Patients with mild anemia are most likely asymptomatic; however, when the anemia worsens, the patients could present with symptoms, such as fatigue, pallor, and dyspnea on exertion. Of note, the presence of anemia increases by twofold the risk of hospitalizations as well as the length of the in-hospital stay[19].

Post-bariatric anemia is in most cases due to iron deficiency, along with vitamin B12 deficiency as a secondary cause. Iron deficiency, expressed by low serum ferritin, occurs in more than 30% of patients after 5 years from surgery, with a similar rate after RYGB and SG, as recently reported by Alexandrou *et al*[20]. Iron-deficiency can be attributed to several causes. Reduced iron absorption due to hypocloridria and the bypassing of the duodenum and proximal jejunum (which are the main sites of iron absorption) are the primary mechanisms that lead to iron deficiency. Post-operative reduction in food intake and changes in food preferences, such as intolerance for meat and dairy products, are important contributory factors.

Measurement of serum ferritin is the best diagnostic test for detecting iron deficiency since it is a more specific and earlier indicator of iron body capacity and becomes abnormal prior to a decrease in serum iron con-centration. For this reason, ferritin and hemoglobin should be periodically monitored in bariatric patients. Current guidelines[21] recommend oral iron supplementation in all operated patients for preventive purposes. However, for the correction of iron deficiency (when iron deficiency sets in), oral supplementation is not sufficient, and intravenous iron administration is required.

Vitamin B12 deficiency is a major cause of anemia in patients who undergo BPD and RYGB, with a prevalence of 19%-35% after 5 years[22]. Purely restrictive procedures are usually not associated with vitamin B12 deficiency. Vitamin B12 deficiency can result from inadequate secretion of intrinsic factor, limited gastric acidity and, above all, the bypassing of the duodenum, which is the main site of vitamin B12 absorption. Since the human body has substantial reserves of vitamin B12, clinical manifestations of a deficit can appear after

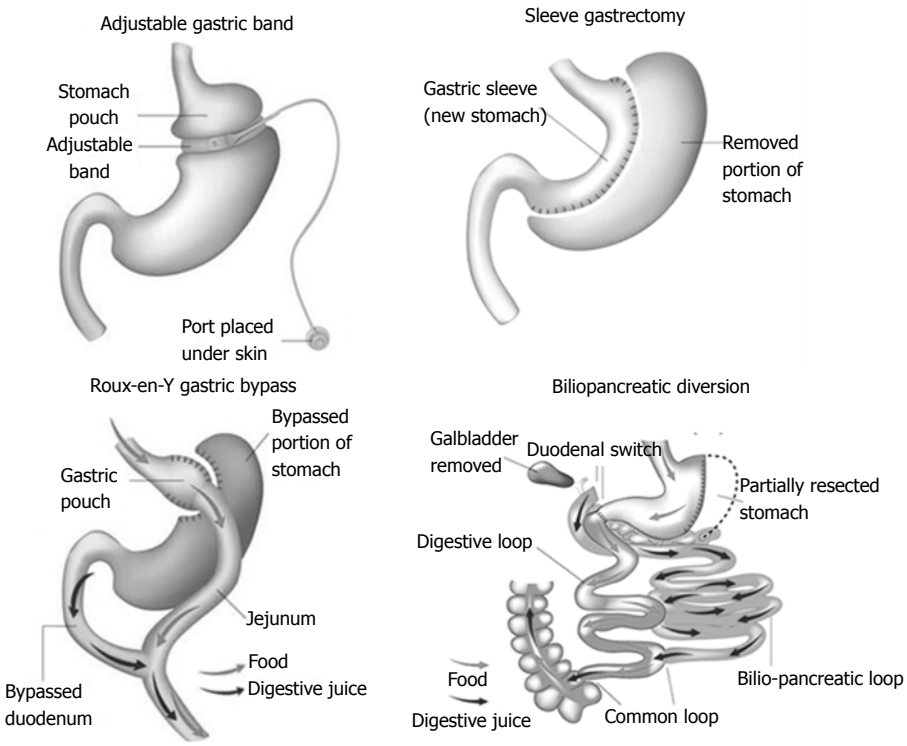

Figure 1 Commonly performed bariatric surgeries. Modified from http://www.bariatric-surgery-source.com/.

a certain time from surgery, when the body stores are depleted to as little as 5%-10%. In addition to anemia, a lack of vitamin B12 can lead to neurological and psychiatric symptoms, including paresthesia, numbness, disturbance of coordination, memory disturbance and, in some instances, dementia. Oral or intramuscular supplementation of vitamin B12 is recommended after malabsorptive procedures, while there is no evidence of benefits after restrictive surgery.

Folic acid deficiency is a potential complication of bariatric procedures that can contribute to anemia. The prevalence of this deficit after both restrictive and malabsorptive procedures ranges from 9% to 39%[23,24]. It can manifest as macrocytic anemia, piastrinopenia, leucopenia, or glossitis. It could cause growth retardation and, in pregnant women, congenital defects (neural tube). Since folate is absorbed throughout the small intestine, the deficiency is primarily induced by a shortage of dietary intake rather than malabsorption. Furthermore, folate deficiency can be aggravated by vitamin B12 deficiency since the latter is necessary for the conversion of inactive methyltetrahydrofolic acid to the active tetrahydrofolic acid. Folate deficiency can be easily corrected by oral supplementation.

Abnormalities of bone metabolism

Bariatric surgery could impact bone metabolism and induce significant changes, such as decreased mechanical loading, calcium/vitamin D malabsorption with secondary hyperparathyroidism, nutritional deprivations, changes in fat mass and alterations in fat- and gut-derived hormones[25-27].

In general, weight loss, achieved through dietary restriction, drugs or bariatric surgery, is associated with a significant reduction in bone mineral density (BMD) and increased bone turnover[28]. In particular, the bone loss reported after non-surgical weight loss is much lower (1%-2%)[29] than that found after bariatric procedures (8%-13%)[30,31] A recent meta-analysis of studies that compare bariatric vs a non-operated control group showed reduced BMD at the femoral neck but not at the lumbar spine[30]. However, it is important to note that the measurement error at the spine BMD is greater than at other sites, which could likely account for this discrepancy. In addition, there is high heterogeneity in the studies analyzed with regard to different surgery procedures, study design (most retrospective), and patient characteristics (ethnicity, sex, menopausal/postmenopausal stage, follow-up length), which could account for the differences between the two sites. Overall, the reductions in the BMD results are greater after malabsorptive or mixed than after restrictive procedures. Studies that compare RYGB and SG have shown a greater bone loss after RYBG than SG, especially at the hip and femoral neck[32]. Accordingly, bone turnover expressed by circulating markers such as CTX, PINP, TRAcP5b was significantly higher after RYGB than after SG[33]. The difference in the BMD between the two procedures could also be related to the different hormonal patterns induced by the two operations. Indeed, there is increasing evidence that many fat- and gut-derived hormones could affect bone health[25,33,34]. In particular, low levels of GIP, ghrelin, amylin, and insulin and high levels of PYY exert negative

effects on the bone mass. In contrast, low serotonin and high GLP-1 levels appear to positively influence the bone metabolism[25]. However, further studies are needed to better define the role of these hormones in the regulation of bone metabolism.

Bariatric surgery is associated with an increased risk of fractures[35,36]. In a population-based study, the cumulative incidence of any new fracture at 15 years was 58% in bariatric patients compared to 24% in non-operated men and women of similar age. The relative risk for any fracture was increased by 2.3-fold both at the traditional osteoporotic (hip, spine, wrist) and at non-osteoporotic sites[35].

Calcium and vitamin D deficiencies are the main factors that are responsible for the accelerated bone loss after bariatric surgery. The incidence of calcium deficiency after surgery is almost 10%[37] and is caused by reduced calcium absorption that results from bypassing the duodenum and proximal jejunum, which are the main sites of absorption. In some cases, calcium deficiency could be exacerbated by low calcium intake due to the intolerance/exclusion of milk products.

The prevalence of hypovitaminosis D after surgery varies between 25% and 73%, depending on the duration of the follow-up and its defining parameters (25-OH-vitamin D < 20 or < 30 ng/mL). It is important to note that hypovitaminosis D exists in a large proportion of patients prior to surgery, with reports that range from 25% to 80%. However, bariatric surgery *per se* affects the vitamin D status[38]. Indeed, similar to calcium deficiency, hypovitaminosis D could be a consequence of fat malabsorption, due to the bypass of the primary absorption sites of liposoluble vitamins in the small intestine[39,40]. In fact, a duodenal surgical bypass decreases cholecystokinin secretion, which results in a reduction in pancreatic lipolytic enzyme secretions and alteration in biliary salts, which in turn leads to an alteration in fat digestion and steatorrhea[24]. In addition, after both malabsorptive and restrictive procedures, reduced intake of dairy products, vomiting, and non-adherence to supplement recommendations could worsen the vitamin D status[39,40].

These are no clear recommendations for vitamin D doses following bariatric surgery, since individual patients could require larger or smaller doses according to the degree of deficit. Current recommendations[21] indicate that at least 5000 IU/d is required to maintain adequate vitamin D levels after RYGB, while higher doses (up to 50000 IU) are required after BPD. Recent studies have suggested that the vitamin D level should be maintained at over 25-30 ng/mL for the effective prevention of osteoporosis and fracture risk. Daily calcium supplementation (preferably as calcium citrate) from 1200 to 2000 mg daily is recommended. It must be considered that oral calcium could interfere with the absorption of some essential minerals such as iron, zinc and copper.

Deficiencies of other vitamins and minerals

Low serum levels of fat-soluble vitamins (vitamin A, K and E) have been found to occur after malabsorptive procedures (BPD and long limb RYGB). However, the available data are largely based on clinical reports and, therefore, are insufficient to estimate the real prevalence of these deficiencies. In two series of studies, the incidence of vitamin A deficiency was 61%-69% at 2-4 year after BPD, with or without duodenal switch[41,42]. In a third series, the incidence was as low as 5% by 4 year[43]. Clinical manifestation of vitamin A deficits are night blindness, xerophthalmia and dry hair.

Low levels of vitamin K have been reported in 50%-60%[42] of patients who underwent BPD or BPD/DS, but no clinical symptoms such as easy bruising, increased bleeding, or clotting alterations were reported.

With regard to the water-soluble vitamins, thiamine (vitamin B1) deficiency can occur in up to 49% of patients after surgery as a result of bypass of the jejunum, where it is primarily absorbed, or in the presence of impaired nutritional intake from persistent, severe vomiting[44]. The early symptoms of thiamine deficiency are nausea and constipation, followed by neurological and psychiatric complications known as Wernicke-Korsakoff syndrome. The prevalence of vitamin C deficiency ranges from 10%-50%[45,46], but it rarely results in manifest clinical signs (poor wound healing, petechiae, bleeding gums).

Although most of the literature focuses on calcium and iron, deficiencies of other essential minerals such as magnesium, zinc, copper, and selenium have been reported in bariatric patients[47]. Essential minerals act as enzymatic cofactors in several biochemical pathways, and therefore, their deficiency could cause variable clinical manifestations that involve neurological, cardiac and gastrointestinal systems. Mineral deficiencies are more common after BPD and RYGB; however, the real prevalence of these disturbances cannot be precisely estimated since most deficiencies can be present already before surgery (see the next paragraph). In addition, for some minerals such as copper and magnesium, the circulating concentrations might not reflect their total body stores, thus leading to underestimation of the real deficit.

Protein malnutrition

Protein malnutrition remains the most severe macronutrient complication associated with malabsorptive surgical procedures. It has been reported in 7%-21% of patients who underwent BPD and is a consequence of poor protein digestion and absorption secondary to altered biliary and pancreatic function[48]. Protein malnutrition can also occur after RYGB, where the Roux limb exceeds 150 cm, with an incidence of 13% at the 2-year follow-up. SG and AGB can lead to protein malnutrition in patients who present maladaptive eating behaviors after surgery, those who avoid protein food sources and those who have protracted vomiting. The clinical signs of protein malnutrition include edema, hearing loss and low serum albumin level (< 3.5 g/dL). Protein malnutrition associated with malabsorptive

procedures causes an annual hospitalization rate of 1% per year and leads to significant morbidity and poor outcomes[49,50]. Monitoring the serum albumin concentration is useful for the evaluation of the protein nutritional state, although the serum protein level often remains in the normal range until late. Measurement of lean body mass by means of dual X–ray absorptiometry or body bioimpedence assessment can be helpful for the evaluation of body composition, although their accuracy appears to be limited in bariatric patients.

According to consensus guidelines[21], the prevention of protein malnutrition requires an average daily protein intake of 60-120 g (1.1 g/kg of ideal body weight), which should be increased by 30% following BPD. Furthermore, great emphasis is posed on regular training and aerobic exercise as being essential to preserving lean mass and especially muscle mass. Patients with severe protein malnutrition should be managed with modular protein supplements that are rich in branch-chain amino acids and, eventually, enteral feeding.

Post-operative weight regain

The regain of the weight lost is one of the main concerns of bariatric patients over the long term. The incidence of this phenomenon is quite variable according to the type of procedure performed, the length of follow-up and, above all, the criteria to define weight regain. Among different definitions, the most widely accepted method refers to a regain of 25%-30% of the maximum weight lost, corresponding to the weight before surgery, with the subtraction of minimum weight or "nadir" after surgery[51-53]. A recent review has shown that the rates of weight regain for SG range from 5.7% at 2 years to 75.6% at 6 years[54]. For RYGB, the percentage of failure to maintain weight loss varies from 7% to 50% of the subjects and tends to be higher in superobese patients[55]. AGB is associated with the largest weight regain (35%-40% of the weight lost), as evidenced in several clinical studies[11,51].

The failure to maintain long-term weight loss has important consequences on the patients' health, including the relapse of obesity-related co-morbidities[56]. Furthermore, it has substantial economic repercussions for the recurrent costs associated with the management of on-going obesity. Therefore, there have been many efforts to understand the biological and psychologic/behavioral bases that underlie this important phenomenon.

One of the major factors responsible for weight regain is the reduction in energy expenditure (EE), which is generally paralleled by the simultaneous loss of lean body mass[57]. Recently, Tam *et al.*[57] showed that EE is significantly reduced 1 year after RYGB (-124 ± 42 kcal/die) as well as after SG (-155 ± 118 kcal/die) compared to the baseline. These findings extend what was already known with diet-induced weight loss and give support to the view that the reduction in EE is a homeostatic mechanism that counteracts a reduction in the caloric intake, which is aimed at preventing excessive weight loss; however, in some conditions, it could favor weight regain.

Another factor that contributes to weight regain is the changes in entero-hormone and appetite regulation[56]. As widely demonstrated, BS is associated with a recovery of the postprandial response of GLP-1, which increases by 3- to 6-fold compared to pre-surgery levels[58]. Interestingly, it has been shown[52] that in patients operated by RYGB, the post-meal response of GLP1 was significantly greater in individuals who maintained weight loss compared to individuals who failed, which suggests that this hormone plays a role in the maintenance of a favorable weight outcome. With regard to ghrelin, the results are quite controversial, with some but not all[59] studies showing greater and more sustained suppression of ghrelin levels in bariatric patients who maintained appropriate weight loss compared to those who regained weight[60,61].

Moreover, mental health disorders, such as depression, alcohol and drug use, and food urges are predictive factors of weight regain[62,63]. Although binge eating is more frequent among obese patients who make recourse to BS (10%-50%), there is no doubt that its persistence after surgery is associated with a minor weight loss and an early weight regain[64].

Beyond all of the above-mentioned factors, the success of bariatric surgery is strongly influenced by the patients' motivation to adhere to a healthier lifestyle, including controlled energy intake and physical activity[65]. In the Swedish Obese Subjects study[66], the reported mean energy intake was 2900 kcal/die before surgery, 1500 kcal/die 6 mo after surgery and approximately 2000 kcal/die 4-10 years after surgery, which demonstrates a progressive increase in calorie intake over the years. These data emphasize dietary counselling and the practice of physical exercise as fundamental measures to prevent weight recidivism.

PRE-OPERATIVE NUTRITIONAL STATE: A CRITICAL FACTOR

It is a common belief that nutritional deficiencies are rare in Western countries due to the availability of low cost and unlimited variety of food supply. However, obese subjects often adopt an unhealthy diet that is rich in high-calorie food with an unbalanced nutritional composition[67,68]. The concomitant presence of high calorie intake and nutrient deficiencies could impact the effectiveness of calorie utilization, which could determine a vicious cycle that leads to further weight gain, depression, eating disorders, metabolic syndrome, fatigue and more[67]. In support of these concepts, a growing number of studies in the literature attest to the frequent occurrence of nutrient and/or vitamin/mineral deficiencies in morbidly obese individuals prior

Table 1 Schedule of biochemical and nutritional assessments for the different bariatric procedures

Assessments	Pre-operative	1 mo	3 mo	6 mo	12 mo	18 mo	24 mo	Annually
MOC DEXA							AGB, SG, RYGB, BPD[1]	AGB3, SG, RYGB, BPD[1]
Calcium	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]
Magnesium	AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	RYGB, BPD[1]		RYGB, BPD[1]	RYGB, BPD[1]
Phosphorus	AGB, SG, RYGB, BPD[1]				AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]
Zinc	AGB, SG, RYGB, BPD[2]		RYGB, BPD[1]	RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]		AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]
Iron	AGB, SG, RYGB, BPD[2]		RYGB, BPD[1]	RYGB, BPD[1]	AGB, SG, RYGB, BPD[2]	RYGB, BPD[1]	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]
Transferrin	AGB, SG, RYGB, BPD[2]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]
Ferritin	AGB, SG, RYGB, BPD[2]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]
Vitamin A	AGB, SG, RYGB, BPD[2]		RYGB, BPD[1]	RYGB, BPD[1]	RYGB, BPD[1]		RYGB, BPD[1]	RYGB, BPD[1]
Vitamin E	AGB, SG, RYGB, BPD[1]				AGB, SG, RYGB, BPD[1]			
Vitamin D	AGB, SG, RYGB, BPD[2]		RYGB, BPD[2]	RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]		AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]
Vitamin B1	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]		AGB, SG, RYGB, BPD[1]	AGB, SG, RYGB, BPD[1]
Vitamin B6	AGB, SG, RYGB, BPD[2]				AGB, SG, RYGB, BPD[1]			AGB[3], SG[3], RYGB[3], BPD[1,3]
Vitamin B12	AGB, SG, RYGB, BPD[1]			AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]
Parathormone	AGB, SG, RYGB, BPD[2]			AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]		AGB, SG, RYGB, BPD[2]	AGB, SG, RYGB, BPD[2]

[1]Useful, including all contents in the space; [2]Recommended, including all contents in the space; [3]Every 2-5 years. AGB: Laparoscopic adjustable gastric banding; SG: Sleeve gastrectomy; RYGB: Roux-en-Y gastric bypass; BPD: Biliopancreatic diversion.

to bariatric surgery, before weight loss and possible surgical-related malabsorption set in.

With regard to the vitamin status, most evidence refers to a 25(OH)vitamin D deficit. Vitamin D insufficiency (< 30 ng/dL) has been reported in approximately 90% of different study populations, and ranges from 65%[69] to 100%[70], while vitamin D deficiency (< 20 ng/dL) is observed in approximately 60% of the patients, ranging from 22%[71] to 83%[72]. The prevalence of severe deficit (< 10 ng/dL) could reach 25%[73]. The degree of deficiency is predicted by the degree of obesity and race, with African Americans being at higher risk[74].

Obese individuals are more likely to be deficient in vitamin D because of the higher volumetric dilution and sequestration of this fat-soluble hormone in the adipose tissue[75]. As the fat mass increases, an individual will require greater amounts of vitamin D (*via* photoproduction from sun exposure, dietary intake, and/or supplementation). Moreover, although there is no difference in the vitamin D_3 production between obese and lean individuals, obese patients show an impaired release of vitamin D_3 from the skin[76]. Genetic variation in the function of the vitamin D binding protein and vitamin D receptor could also influence the 25(OH)D levels, with some studies suggesting a higher frequency of the poorer functioning forms in obesity[77,78].

The prevalence of vitamin B12 deficiency in patients scheduled for BS is reported in approximately 18% of patients. Similarly, low levels of vitamin B1 (thiamine) are reported in up to 20% of bariatric candidates. Few studies have assessed the vitamin C status in bariatric candidates, with a prevalence that ranges from 15%[69] to 33%[79]. With regard to vitamins A and E, their deficiencies are less frequent[69,73]. In particular, vitamin A has been found to be inversely associated with BMI, age and number of comorbidities[73]. This finding most likely occurs because low vitamin A levels are related to increased oxidative stress, insulin resistance, impaired glucose metabolism, cancers, and age-related macular degeneration[80], all of which are commonly associated with morbid obesity.

Among the minerals, iron deficiency is the most common and ranges from 20% to 47%[81]. Iron and ferritin deficiency and iron-deficiency anemia are more frequent in younger patients (< 25 years) than in older patients and in women than in men, although this finding is not confirmed in all studies[82]. Iron deficiency in obese patients is likely related to the negative impact that chronic inflammation exerts on iron homeostasis. In particular, there is evidence that cytokines (TNFα and

IFNγ) can induce the apoptosis of erythroid progenitor cells and increase hepcidin levels, which leads in turn, to reduced intestinal iron absorption and reduced bioavailability[83].

The prevalence of zinc deficiency prior to bariatric surgery amounts to 10.2%[84-86]. Interestingly, some studies have shown an inverse association of zinc levels, with C-reactive protein highlighting the adverse influence of systemic low-grade inflammation on the zinc status[84].

Overall, the high prevalence of pre-surgery nutritional deficiencies in bariatric candidates supports the need for a careful pre-operative evaluation of the nutritional status, to assess and adequately correct the pre-existing deficits.

CONCLUSION

Nutritional deficiencies represent a relevant long-term clinical problem in patients who underwent bariatric surgery as a result of modifications to the gastrointestinal anatomy and physiology, which could impact macro- and micro-nutrient absorption. Therefore, the best practices guidelines[21] highly recommend regular metabolic and nutritional monitoring after bariatric surgery, which frequency varies according to the type of procedure. In light of the high prevalence of nutrient deficiencies even prior to surgery, the current Guidelines also underscore the need for a complete pre-surgery nutritional assessment in all candidates for bariatric surgery. The schedule of the biochemical and nutritional monitoring for the different procedures is reported in Table 1. Although there are few studies with long-term nutritional follow-up, there is general agreement that nutritional assessments should be performed throughout life; furthermore, multivitamin and calcium supplementation with added vitamin D is recommended for all weight-loss surgery patients. In conclusion, nutritional surveillance is an essential component of the management of bariatric patients for the following reasons: (1) increases the patients' adherence to healthy dietary habits and appropriate supplementation regimens; (2) prevents the risk of weight regain; (3) facilitates the detection of possible nutritional deficiencies that could develop despite medical therapy; and (4) contributes to maintaining a good quality of life.

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ORIGINAL ARTICLE

Retrospective Study

Reproductive disturbances among Saudi adolescent girls and young women with type 1 diabetes mellitus

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Abstract

AIM

To identify reproductive disturbances among adolescent girls and young women with type 1 diabetes mellitus (T1DM) in Saudi Arabia.

METHODS

This cross sectional study was conducted among 102 female with T1DM, (aged 13-29 years) who attended the Diabetes Clinic at Diabetes Treatment Center, Prince Sultan Military Medical City, Saudi Arabia between April 2015 to March 2016. Clinical history, anthropometric characteristics and reproductive disturbance were collected through a questionnaire.

RESULTS

Of 102 patients included in this analysis, 26.5% (27/102) were reported that they experienced an irregular menses. Of these patients, when compared to whose diabetes was diagnosed before menarche (35.4%, 17/48), patients diagnosed with diabetes after menarche (18.5%, 10/54) showed significantly less irregular menses (difference 16.9%, $P = 0.04$). Similarly, compared to patients diagnosed with diabetes prior to menarche (mean age 12.9 years; $n = 48$), patients diagnosed with diabetes after menarche (mean

age 12.26 years; *n* = 54) were found to have 0.64 years delay in the age of menarche (*P* = 0.04). Among the studied patients, 15.7% (16/102) had polycystic ovary syndrome (PCOS). Of these PCOS patients, 37.5% (6/16) had irregular menses, 6.3% (1/16) had Celiac disease, 37.5% (6/16) had Hashimoto thyroiditis and 18.7% (3/16) had acne.

CONCLUSION

More than one fourth of the study population with T1DM experiencing an irregular menses. Adolescent girls and young women diagnosed with diabetes prior to menarche showed higher menstrual irregularity and a delay in the age of menarche.

Key words: Type 1 diabetes; Reproductive disturbances; Polycystic ovarian syndrome; Premature ovarian failure; Menarche; Saudi Arabia

Core tip: The present study found more than one fourth of the adolescent girls and young women with type 1 diabetes experiencing an irregular menses. Adolescent girls and young women diagnosed with diabetes prior to menarche reported 16.9% higher menstrual irregularity and 0.64 years delay in the age of menarche.

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INTRODUCTION

The last few decades have shown a trend of a steady increase in the incidence of type 1 diabetes mellitus (T1DM) patients in most parts of the world[1-3]. Research has shown a rise in the incidence of T1DM in Saudi Arabia in the preceding 30 years as well as in the prevalence of T1DM among the children and adolescents in the Kingdom to be 109.5 per 100000, a figure higher than that of several advanced countries[4-6].

T1DM is a chronic autoimmune disease that represents a multi-faceted challenge to normal reproductive function throughout life. Despite the improvements in diabetes therapy, adolescent girls and young women with T1DM still face frequent disturbances in the reproductive system including infertility, delayed onset of puberty and menarche, menstrual irregularities (especially oligomenorrhoea) and premature ovarian failure (POF)[7-9]. Such females show a tendency, on average, to attain menarche a little later in life than non-diabetic women[10]. At the other extreme, diabetic women tend to experience menopause marginally sooner[8,11]. Furthermore, women with T1DM exhibit a high degree of hyperandrogenic disorders, like polycystic ovary syndrome (PCOS) and hirsutism[12]. Despite the previous, PCOS is commonly linked with conditions driven by insulin resistance, such as type 2 diabetes mellitus (T2DM)[13]. However, several studies demonstrated that T1DM patients may also experience insulin resistance, especially in obese individuals, and even PCOS[12,14].

Compared to studies performed in the developed countries, limited literature are available in Saudi Arabia on reproductive disturbances among adolescent girls and young woman due to the lack of appropriate studies performed in these specific aspects. Therefore, the current work was done as a cross-sectional study to identify the reproductive disturbances among adolescent girls and young women with T1DM in a tertiary care center, specifically attempting to isolate the factors that can cause such abnormalities.

MATERIALS AND METHODS

Study design and setting

This cross sectional study was conducted among 102 female with T1DM (aged 13-29 years) who attended the Diabetes Clinic at Diabetes Treatment Center, Prince Sultan Military Medical City (PSMMC), Saudi Arabia between April 2015 to March 2016. The PSMMC is a 1200-bed, tertiary medical center in Riyadh, Saudi Arabia, with almost 40000 annual admissions (950000 active patients files) per year from different region of the country.

Criteria for selection of patients

The participants were conveniently selected according to their availability during their routine visit to the outpatient clinics. All patients provided written informed consent to participate the study, for adolescent patients consent form were collected from their parents/legal guardians.

Adolescent patients with T1DM and young women aged 13-29 years and Saudi nationals were included in the study, while patients with T2DM, double diabetes (expressing features resulting from both type 1 diabetes and type 2 diabetes), maturity onset diabetes of young (MODY), pregnant and patients using oral contraceptive pills were excluded. Also, patients with the other conditions with similar phenotypical characteristics to those associated with PCOS such as Hyperprolactinaemia states, Cushing's syndrome, Acromegaly, Congenital adrenal hyperplasia, thyroid disorders, adrenal tumors (adrenal carcinoma, adrenal adenoma), ovarian tumors, androblastomas (Sertoli–Leydig cell tumors), granulosa cell tumors, Sertoli cell tumors, Hilus cell tumors, primitive neuro-ectodermal tumors (PNET) and HAIR-AN syndrome were excluded.

Data collection and definitions

Anthropometric characteristics and a detailed clinical history were obtained through a questionnaire. A

complete physical examination was performed for all patients. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of height in meters (BMI; kg/m^2) and BMI z score (adjusted for child age and gender). The *z* score (or SD score) was calculated as per the formula (Xi-Mx)/SD, where Xi is the actual measurement, Mx is the mean value for that age and gender, and SD is the standard deviation corresponding to that age and gender [15].

History of recurrent diabetic ketoacidosis (RDKA) (defined as three or more episodes occurring within a period of four years as visiting the accident and emergency room or admitted in hospital), dyslipidemia, Hashimoto thyroiditis, Celiac disease and premature ovarian failure (POF) were also collected[16]. All patients were also screened for diabetic complications (neuropathy and retinopathy and nephropathy) if the duration of their diabetes 5 years or more.

Menstrual disturbances were recorded based on verbal information provided by the patients. Oligomenorrhea was defined by 3 or more cycles with a length of more than 36 d in the previous year, and amenorrhea was defined by lack of vaginal bleeding for the last 3 mo. Hirsutism was defined as excess terminal (thick pigmented) body hair in an androgen-dependent pattern, and which is commonly noted on the upper lip, chin, periareolar area, in the midsternum, and along the linea alba of the lower abdomen. It was estimated according to the modified Ferriman-Gallwey scale score of 8 or more, which was determined by a single experienced observer[17]. The presence of acne was also evaluated. The puberty was appreciated according to the Tanner Stage[18]. The POF was defined as the development of irregular menses or amenorrhea before the age of 40 years in association with follicle-stimulating hormone (FSH: IU/L) concentrations in the postmenopausal range (as defined by the measuring laboratory). Polycystic ovaries were diagnosed by pelvic or intravaginal sonography according to the Rotterdam consensus criteria. PCOS was defined by the presence of two out of three of the following criteria: (1) Oligo- and/or anovulation (irregular menses or amenorrhea); (2) Clinical and/or biochemical signs of hyperandrogenism; and (3) Polycystic ovaries (by pelvic ultrasound, include the presence of 12 or more follicles in each ovary measuring 2 to 9 mm in diameter and/or increased ovarian volume (>10 mL; calculated using the formula 0.5 × length × width × thickness). One ovary fitting this definition is sufficient to define polycystic ovarian morphology (PCOM) and exclusion of other etiologies[19].

The glycosylated haemoglobin A1c (HbA1c) testing was performed in our laboratory using a method that is The National Glycohaemoglobin Standardization Program (NGSP), United States certified and standardized to the Diabetes Control and Complications Trial (DCCT) assay[20]. Total testosterone level was done for all the patients (normal level 0.69 to 2.1 nmol/L). Plasma testosterone was analyzed using Elecsys Testosterone Ⅱ from Roche company where the electrochemiluminescence immunoassay "ECLIA" was intended for use on Elecsys and cobas e immunoassay analyzers. Regarding the testosterone level, 0.69 to 2.1 nmol/L considered as normal and testosterone > 2.1 mmol/L considered as high testosterone level.

In patients with irregular menses and no clinical or biochemical features of hyperandrogenism, we performed pelvic ultrasound to study the morphology of the ovaries. Pelvic ultrasound was also performed in patients with amenorrhea.

Statistical analysis

Data analysis was carried out using Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, United States) and Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, IL, United States). In addition to the descriptive analysis, χ^2 test (for categorical variables) and independent *t* test (for continuous variables) were also performed to identify variables associated with reproductive disturbances before and after diagnosis menarche and patients with PCOS and without PCOS. Continuous variables are represented as mean values ± SD, while categorical variables are expressed as frequencies and percentages. A *P*-value of < 0.05 was considered as statistically significant.

RESULTS

The overall mean of the clinical parameters of the study population were as follows: Age 18.26 ± 4.05 (range 13-29 years), age at diagnosis of diabetes 11.5 ± 3.96 (range 1-21 years), duration of T1DM 6.8 ± 5.35 years (range 1-28 years), age at menarche 12.56 ± 0.96 (range 9-16 years), BMI 23.54 ± 3.3 kg/m^2 (range 17.8-34.4) and HbA1c was 9.23 ± 1.92 (range 6-16).

Clinical and anthropometric characteristics of the study population are shown in Table 1. Among the studied population, 41.2% (42/102) possess family history of DM; 15.7% (16/102) had PCOS; 1% (1/102) had POF; and 2.95% (3/102) had amenorrhea. The study also found that 26.5% (27/102) of patients had irregular menses; 18.6% (19/102) had acne; 6.9% (7/102) had Celiac disease; 33.3% (34/102) had Hashimoto thyroiditis; 13.7% (14/102) had dyslipidemia, 24.5% (25/102) had hirsutism;12.7% (13/102) had RDKA and 11.8% (12/102) had diabetes complication (neuropathy and retinopathy and none of our patients had nephropathy).

Majority of the study population are in teen age group 13-19 (69.6%, 71/102; mean = 15.9). Among the 71 teenagers, 2.8% (2/71) underweight, 26.8% (19/71) overweight, 2.8% (2/71) obese and 67.6% (48/71) were normal. The mean age of the young women (20-29 years, *n* = 31) was 23.5 years. Among the young women, 3.2% (1/31) underweight, 48.4% (15/31) overweight, 3.2% (1/31) obese and 45.2% (14/31) were normal.

The factors associated with menarche before and after diagnosis of diabetes are shown in Table 2. A total

Table 1 Clinical and anthropometric characteristics of the study population

Variables	Frequency (n = 102)	%
Age (yr)		
13-19	71 (mean age 15.9 ± 2.72)	69.6
20-29	31 (mean age 23.5 ± 2.41)	30.4
Family History of diabetes		
No	60	58.8
Yes	42	41.2
Polycystic ovarian syndrome		
No	86	84.3
Yes	16	15.7
Recurrent diabetic ketoacidosis		
No	89	87.3
Yes	13	12.7
Complications of diabetes		
No	90	88.2
Yes	12	11.8
Age of diagnosis (yr)		
1-10	34 (mean 7.1 ± 2.85)	33.3
11-21	68 (mean 13.8 ± 1.99)	66.7
Age of menarche (yr)		
9-11	5	4.9
12	50	49
13-16	47	46.1
Menses		
Irregular	27	26.5
Regular	75	73.5
Premature ovarian failure		
No	101	99
Yes	1	1
Celiac disease		
No	95	93.1
Yes	7	6.9
Hashimoto thyroiditis		
No	68	66.7
Yes	34	33.3
Dyslipidemia		
No	88	86.3
Yes	14	13.7
Hirsutism		
No	77	75.5
Yes	25	24.5
Acne		
No	83	81.4
Yes	19	18.6
Pelvic ultrasound		
Not done	67	66.7
Small ovaries	3	2.9
Normal	11	10.8
PCOS	21	20.6
Testosterone level		
Normal	77	75.5
High	25	24.5
Hemoglobin A1C		
≤ 7%	13 (mean 6.67 ± 0.57)	12.7
> 7%	89 (mean 9.57 ± 1.78)	87.3

PCOS: Polycystic ovary syndrome.

of 47.1% (48/102) patients were diagnosed as having diabetes before menarche and 52.9% (54/102) were diagnosed as having diabetes after menarche. Age, duration of DM and acne showed statistically significant differences among the two groups. Similarly, when compared with those identified with diabetes before menarche (9.73%), the girls diagnosed with diabetes post menarche (8.78%) reported lower HbA1c levels (P = 0.007).

Figure 1 shows the percentage differences of irregular menses among patients diagnosed with diabetes before and after menarche. Compared to whose diabetes was diagnosed before menarche 35.4% (17/48), patients diagnosed with diabetes after menarche 18.5% (10/54) showed less irregular menses (differences 16.9%, P = 0.04). Similarly, compared with

Table 2 Factors associated with menarche before and after diagnosis of diabetes

Variables	Diabetes diagnosed before menarche (n = 48), n (%)	Diabetes diagnosed after menarche (n = 54), n (%)	P value
Age (yr)			
13-19 (n = 71)	38 (53.5)	33 (46.5)	0.038
20-29 (n = 31)	10 (32.3)	21 (67.7)	
Family history of diabetes			
No (n = 60)	28 (46.7)	32 (53.3)	0.542
Yes (n = 42)	20 (47.6)	22 (52.4)	
Polycystic ovarian syndrome			
No (n = 86)	42 (48.8)	44 (51.2)	0.289
Yes (n = 16)	6 (37.5)	10 (62.5)	
Recurrent diabetic ketoacidosis			
No (n = 89)	42 (47.2)	47 (52.8)	0.591
Yes (n = 13)	6 (46.2)	7 (53.8)	
Complications of diabetes			
No (n = 90)	41(45.6)	49 (54.4)	0.299
Yes (n = 12)	7 (58.3)	5 (41.7)	
Menses			
Irregular (n = 27)	17 (63)	10 (37)	0.04
Regular (n = 75)	31 (41.3)	44 (58.7)	
Premature ovarian failure			
No (n = 101)	48 (47.5)	53 (52.5)	
Yes (n = 1)	0	1 (100)	0.529
Celiac disease			
No (n = 95)	44 (46.3)	51(53.7)	0.434
Yes (n = 7)	4 (57.1)	3 (42.9)	
Hashimoto thyroiditis			
No (n = 68)	31 (45.6)	37 (54.4)	0.416
Yes (n = 34)	17 (50)	17 (50)	
Dyslipidemia			
No (n = 88)	42 (47.7)	46 (52.3)	0.482
Yes (n = 14)	6 (42.9)	8 (57.1)	
Hirsutism			
No (n = 77)	37 (48.1)	40 (51.9)	0.452
Yes (n = 25)	11 (44)	14 (56)	
Acne			
No (n = 83)	45 (54.2)	38 (45.8)	0.002
Yes (n = 19)	3 (15.8)	16 (84.2)	
Pelvic ultrasound			
Not done (n = 67)	31 (46.3)	36 (53.7)	0.103
Small ovaries (n = 3)	3 (100)	0	
Normal (n = 11)	7 (63.6)	4 (36.4)	
Polycystic ovary syndrome (n = 21)	7 (33.3)	14 (66.7)	
Testosterone level			
Normal (n = 77)	40 (51.9)	37 (48.1)	0.065
High (n = 25)	8 (32)	17 (68)	
Variables	mean ± SD	mean ± SD	P value
Body mass index	23.83 ± 0.5	23.28 ± 0.3	0.123
Duration of diabetes	9.13 ± 0.8	4.74 ± 0.5	0.011
Age at diabetes diagnosis	8.42 ± 0.4	14.3 ± 0.2	0
Insulin dose unit/kg	0.84 ± 0.23	0.88 ± 0.23	0.794
Hemoglobin A1c	9.73 ± 0.3	8.78 ± 0.2	0.007
Body mass index	23.83 ± 0.5	23.28 ± 0.3	0.123

Categorical variables analyzed by χ^2 test and the continuous variables analyzed by t-test. $P < 0.05$ considered as significant.

patients diagnosed with diabetes prior to menarche (mean age 12.9 years), patients diagnosed with diabetes post menarche (mean age 12.26 years) were found to have 0.64 years less in the age of menarche (P = 0.04) (Figure 2).

Results displayed as patients with PCOS (15.7%; 16/102) and without PCOS (84.3%; 86/102) are shown in Table 3. Out of 71 teenagers and out of 31 young women 15.5% (11/71) and 16.1% (5/31) had PCOS respectively. Of these PCOS patients, 37.5% (6/16) had irregular menses, 6.3% (1/16) had Celiac disease, 37.5% (6/16) had Hashimoto thyroiditis and 18.7% (3/16) had acne. There were no significant differences were observed among patients with PCOS and without PCOS except the variables family history (P = 0.001) and BMI (P = 0.008).

DISCUSSION

Reproductive disturbance has long been recognized

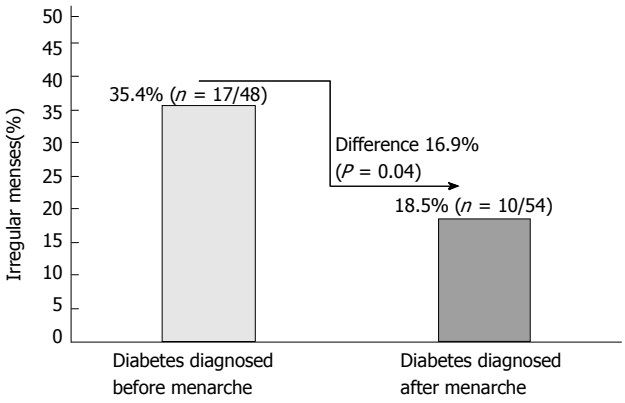

Figure 1 Irregular menses among girls/young women diagnoses with diabetes before (*n* = 48) and after menarche (*n* = 54).

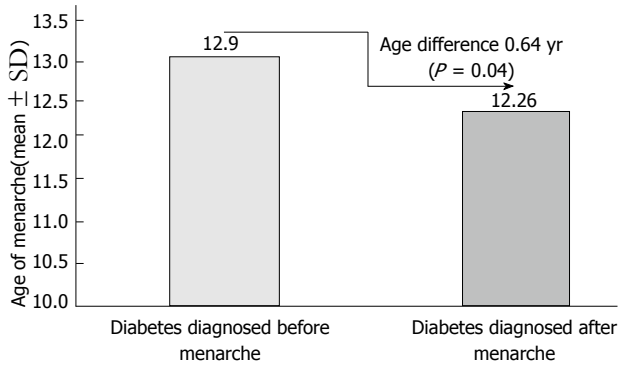

Figure 2 Mean age of menarche among girls/young women (*n* = 102) diagnoses with diabetes before and after menarche.

as a prevalent problem among girls and young women with T1DM[21]. In the present study, we found that approximately one fourth (26.5%) of the study population experiencing an irregular menses. An earlier study reported that approximately one third of young women with T1DM suffer some kind of menstrual dysfunction[21]. However, other studies reported that the rates of menstrual irregularity are 50% higher among patients with T1DM than those without T1DM[10,22]. Furthermore, it is well demonstrated that T1DM decreased physical, psychological well-being (depression and diabetes-related distress) and decreased the quality of life of adolescent girls and young women[23]. Such disturbances combined with other dysfunctions that could influence the reproductive system, are highly significant for type 1 diabetic adolescent girls and young women; in particular, those diagnosed prior to puberty often have peripubertal disturbances[25-27].

A growing number of studies have investigated that globally, a secular trend of younger age at menarche has been well recognized[28-30]. The mean age of menarche varies from about 12.5 years in the United States to 12.72 in Canada, 12.9 in the United Kingdom and 13.06 years in Iceland[28-30]. A study done on girls in Istanbul, Turkey, identified the median age at menarche to be 12.74 years[31]. In Saudi Arabia, the age of menarche shows considerable declining patterns. In the past decade, a study reported that the mean age at menarche among Saudis was 13.05[32]. However, more recent data from a study in 2015 indicated that the mean age of menarche for Saudi school going girls was 11.5 ± 1.48 years[33]. The present study revealed the mean age of menarche was 12.56 ± 0.96 among adolescent girls and young women with T1DM, which is higher than previous study findings (11.5 ± 1.48 years) among the normal school going girls in Saudi Arabia[33].

Similar to others, this study also identified delayed menarche among girls with T1DM[34,35]. The results from this present study demonstrated, that when those diagnosed with diabetes prior to menarche (12.26 years), experienced a delay (0.64 years) in the mean age of onset of menstruation compared with girls diagnosed after menarche. Other studies reported a delay of about one year in girls with T1DM at the age of menarche if diabetes mellitus onset was before puberty[25-27]. Kjaer, similar to the earlier findings of Bergqvist and Burkart, reported that should diabetes develop during childhood, menarche is often delayed[25]. However, age of menarche is comparable to that of the non-diabetic controls if diabetes mellitus onset occurred post puberty. This implies that menarche is not influenced by a genetic predisposition to diabetes but is possibly affected by the presence of clinical diabetes and diabetic metabolic disturbances, specifically the high HbA1c levels and duration of diabetes[26,27]. In the present study, when compared to patients who diagnosed as having diabetes after menarche (9.73 ± 0.3), patients who diagnosed as having diabetes before menarche (7.8 ± 0.2) had significantly lower HbA1c. Despite diagnostic and therapeutic advances of recent decades, delay at menarche and high prevalence of menstrual irregularity is still detected among adolescent females with T1DM[10,36]. The present study shows, that compared with those diagnosed with diabetes before menarche (35.4%), the girls diagnosed with diabetes after menarche (18.5%) experience less menstrual disturbance. Similarly, when compared with those identified with diabetes before menarche (9.73%), the girls diagnosed with diabetes post menarche (8.78%) had lower HbA1c levels. Studies also indicated that age at diabetes diagnosis and the HbA1c level were the risk factors for patients diagnosed prior menarche, which later in life resulted in the impairment of crucial life processes, including disturbances in menstruation and fertility, sexual and urinary tract dysfunctions[37]. An epidemiological study investigating menarche and menstrual disturbances, demonstrated menstrual dysfunction in 21.6% of women with T1DM compared with 10.8% in the nondiabetic controls, in 245 insulin-treated diabetic women and 253 healthy women. Therefore, it was evident that menstrual dysfunction occurred nearly twice as often in women with T1DM compared with the non-diabetic controls[26].

The fact that PCOS, which occurs quite frequently in women in the reproductive age, is related to reproductive and metabolic dysfunctions[38]. One of the

Table 3 Factors associated with polycystic ovarian syndrome *n* (%)

Variables	No PCOS (*n* = 86)	PCOS (*n* = 16)	*P* value
Age (yr)			
13-19 yr (*n* = 71)	60 (84.5)	11 (15.5)	0.574
20-29 yr (*n* = 31)	26 (83.9)	5 (16.1)	
Family History of diabetes			
No (*n* = 60)	57 (95)	3 (5)	0.001
Yes (*n* = 71)	29 (69)	13 (31)	
Recurrent diabetic ketoacidosis			
No (*n* = 89)	75 (84.3)	14 (15.7)	0.43
Yes (*n* = 13)	11 (84.6)	2 (15.4)	
Complications of diabetes			
No (*n* = 90)	79 (87.8)	11 (12.2)	0.092
Yes (*n* = 12)	7 (58.3)	5 (41.7)	
Menses			
Irregular (*n* = 27)	21 (78)	6 (16.7)	0.214
Regular (*n* = 75)	65 (87)	10 (15.4)	
Premature ovarian failure			
No (*n* = 101)	85 (84.2)	16 (15.8)	0.843
Yes (*n* = 1)	1 (100)	0	
Celiac disease			
No (*n* = 95)	80 (84.2)	15 (15.8)	0.698
Yes (*n* = 7)	6 (85.7)	1 (14.3)	
Hashimoto thyroiditis			
No (*n* = 68)	58 (85.3)	10 (14.7)	0.453
Yes (*n* = 34)	28 (82.4)	6 (17.6)	
Dyslipidemia			
No (*n* = 88)	76 (86.4)	12 (13.6)	0.15
Yes (*n* = 14)	10 (71.4)	4 (28.6)	
Hirsutism			
No (*n* = 77)	62 (80.5)	15 (19.5)	0.054
Yes (*n* = 25)	24 (96)	1 (4)	
Acne			
No (*n* = 83)	70 (84.3)	13 (15.7)	0.612
Yes (*n* = 19)	16 (84.2)	3 (15.8)	
Pelvic ultrasound			
Not done (*n* = 67)	54 (80.6)	13 (19.4)	0.317
Small ovaries (*n* = 3)	2 (66.7)	1 (33.3)	
Normal (*n* = 11)	10 (91)	1 (9)	
PCOS (*n* = 21)	20 (95.2)	1 (4.8)	
Testosterone level			
Normal (*n* = 77)	62 (80.5)	15 (19.5)	0.054
High (*n* = 25)	24 (96)	1 (4)	
Variables	mean ± SD	mean ± SD	*P* value
Body mass index	23.2 ± 2.86	25.3 ± 4.77	0.008
Duration of diabetes	6.59 ± 5.16	7.94 ± 6.45	0.53
Age at diabetes diagnosis	11.6 ± 3.82	10.8 ± 4.69	0.227
Age of menarche	12.5 ± 1	12.63 ± 0.69	0.118
Insulin dose unit/kg	0.8 ± 0.24	0.83 ± 0.20	0.493
Hemoglobin A1c	9.23 ± 1.98	9.19 ± 1.64	0.516

Categorical variables analyzed by χ^2 test and the continuous variables analyzed by *t*-test. $P < 0.05$ considered as significant. PCOS: Polycystic ovarian syndrome.

main characteristics of PCOS[39,40] is obesity, ranging from 12.5%[41] to 100%[42], with a total estimated prevalence of 49%[43], as reported in a recent meta-analysis[44]. Obesity may exacerbate the metabolic and reproductive disorders linked to this syndrome[45], like insulin resistance, dyslipidemia, and metabolic syndrome[38]. The meta-analysis revealed that women with PCOS exhibit higher levels of triglycerides (TG), LDL-cholesterol and total cholesterol (TC), and lower levels of HDL-cholesterol when compared with the controls, irrespective of BMI[46]. This present study showed a significant difference in the BMI between those with PCOS (mean BMI 25.3) and those without PCOS (mean BMI 23.2). Similarly, a family history of DM also revealed a significant difference between those with PCOS and those without the condition, a fact supported by earlier studies which recorded significantly increased numbers of women with a positive family history of diabetes among those with PCOS[47].

This study has few limitations, mainly, it was performed in a single center examining only a specified number of risk factors, there was no control group with which to compare the study population, patients were taking oral contraceptive pills were excluded which can

lead to underestimation of a reproductive disturbances proportion of female with T1DM especially PCOS and finally the details regarding the pelvic ultrasound report that in most cases does not include the measurement of the ovaries but defined only as small or normal. More research is necessary to address the limitation identified in this work. However, this study presents pertinent information on reproductive disturbance among Saudi adolescent girls and young women with T1DM.

In conclusion, more than one fourth of the study population with T1DM experiencing an irregular menses. Adolescent girls and young women diagnosed with diabetes prior to menarche reported higher menstrual irregularity and a delay in the age of menarche. More studies are required to confirm these findings among T1DM patients from different ethnic backgrounds.

COMMENTS

Background

Many studies have reported frequent disturbances in the reproductive system including infertility, delayed onset of menarche, menstrual irregularities and premature ovarian failure, in adolescent girls and young women with type 1 diabetes. Such females show a tendency, on average, to attain menarche a little later in life than non-diabetic women. Further, studies have found that there is a delay in the age of menarche and higher menstrual irregularity among type 1 diabetes if the onset of diabetes occurs before or near the onset of menarche and that this delay increases with poor glycemic control.

Research frontiers

Reproductive disturbance has long been recognized as a prevalent problem among girls and young women with type 1 diabetes mellitus (T1DM). However, compared to studies performed in the developed countries, limited literatures are available in Saudi Arabia on reproductive disturbances among adolescent girls and young woman due to the lack of appropriate studies performed in these specific aspects.

Innovations and breakthroughs

The authors found more than one fourth of the adolescent girls and young women with type 1 diabetes experiencing an irregular menses. Adolescent girls and young women diagnosed with diabetes prior to menarche reported 16.9% higher menstrual irregularity and 0.64 years delay in the age of menarche.

Applications

A better understanding of the nature, evolution and underlying mechanisms of the reproductive disturbances will help to develop the improved diagnostic and therapeutic strategies for an imperative set of co-morbidities disturbing the adolescent girls and young women with type 1 diabetes.

Peer-review

This is a well-written manuscript on an interesting and important clinical issue namely the frequency of irregular menstruation and amenorrhea in young women and teenagers with type 1 diabetes.

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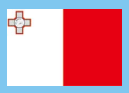

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Gut-brain crosstalk regulates craving for fatty food

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Abstract

Patients undergoing Roux-en-Y gastric bypass (RYGB) surgery elicit striking loss of body weight. Anatomical restructuring of the gastrointestinal (GI) tract, leading to reduced caloric intake and changes in food preference, are thought to be the primary drivers of weight loss in bariatric surgery patients. However, the mechanisms by which RYGB surgery causes a reduced preference for fatty foods remain elusive. In a recent report, Hankir *et al* described how RYGB surgery modulated lipid nutrient signals in the intestine of rats to blunt their craving for fatty food. The authors reported that RYGB surgery restored an endogenous fat-satiety signaling pathway, mediated *via* oleoylethanolamide (OEA), that was greatly blunted in obese animals. In RYGB rats, high fat diet (HFD) led to increased production of OEA that activated the intestinal peroxisome proliferation activator receptors-α (PPARα). In RYGB rats, activation of PPARα by OEA was accompanied by enhanced dopamine neurotransmission in the dorsal striatum and reduced preference for HFD. The authors showed that OEA-mediated signals to the midbrain were transmitted *via* the vagus nerve. Interfering with either the production of OEA in enterocytes, or blocking of vagal and striatal D1 receptors signals eliminated the decreased craving for fat in RYGB rats. These studies demonstrated that bariatric surgery led to alterations in the reward circuitry of the brain in RYGB rats and reduced their preference for HFD.

Key words: Roux-en-Y gastric bypass surgery; Dietary lipids; Dopamine D1 receptors; Peroxisome proliferator activated receptor-alpha; Oleoylethanolamide

Core tip: The mechanisms underlying a massive and sustained body weight loss after gastric bypass surgery remain poorly understood. Hankir *et al* describe how a fat-satiety signaling pathway that was greatly blunted in obese rats could be restored by Roux-en-Y gastric bypass (RYGB) surgery. The authors have demonstrated that RYGB rats on high fat diet (HFD) elicited an increased production of oleoylethanolamide (OEA) and activation of PPARα that led to a surge in dopamine release and

activation of D1 in the dorsal striatum. The enhanced dopamine neurotransmission evoked by OEA was obligatorily dependent on intact vagus nerve that had no effect on the production of OEA in the small intestine. The heightened dopamine neurotransmission in the midbrain of RYGB rats was linked to their decreased preference for HFD. These elegant studies have provided a compelling mechanism by which RYGB surgery led to altered gut-brain communication to modify the reward circuitry involved in food preference and obesity. These observations have important clinical implications for the amelioration obesity and its pathological consequences.

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COMMENTARY ON HOT TOPICS

Patients undergoing Roux-en-Y gastric bypass (RYGB) surgery elicit striking loss of body weight. Anatomical re-structuring of the gastrointestinal (GI) tract, leading to reduced caloric intake and changes in food preference, are thought to be the primary drivers of weight loss in bariatric surgery patients. However, the mechanisms by which RYGB surgery causes a reduced preference for fatty foods remain elusive. In a recent report, Hankir *et al*[1] described how RYGB surgery modulated lipid nutrient signals in the intestine of rats to blunt their craving for fatty food. These studies have provided a compelling mechanism by which RYGB surgery led to altered gut-brain communication to modify the reward circuitry involved in food preference and obesity.

Obesity associated diseases represent a looming global healthcare crisis of the 21st century. Although a collusion of genetic, behavioral and environmental factors regulates body mass in humans, its two key drivers are ready accessibility of calorically-dense foods and sedentary lifestyle[2]. Studies aimed at uncovering the Mendelian causes of obesity have revealed that genes encoding leptin or its receptor, or pro-opiomelanocortin and melanocortin-4 receptor are most commonly mutated in genetically obese patients[3,4]. Detailed investigations of monogenic obesity have yielded important insights into the cellular and molecular mechanisms that underpin morbid obesity. However, a key insight emerging from these studies is that a world-wide prevalence of monogenic obesity is rare and a vast majority of cases of obesity are polygenic. The polygenic nature of severe obesity has been most clearly unraveled by genome-wide association studies (GWAS), aimed at deciphering a link between single nucleotide polymorphisms (SNPs) and body mass index (BMI)[5,6]. For example, a recent GWAS in European adults revealed a strong link between 32 common SNPs and severe obesity; it was also notable that individually, none of the 32 SNPs showed significant association with BMI[7].

Severely obese patients, regardless of whether their obesity is monogenic or polygenic in origin, usually engage in overeating and show a preference for fatty diet[8]. Similar to humans, obese rodents also prefer high fat diet (HFD). Although the mechanisms of eating behavior or food preference are poorly defined, they are dependent on reciprocal gut-brain communication, as shown by functional magnetic resonance imaging and positron emission tomography (PET) imaging studies[9-12].

Nutrient-derived signaling pathways play a central role in sensing the hedonic value of food and satiety. Fat, in addition to being a key macronutrient, is vitally involved in gut-brain signaling. The intake and metabolism of fat are closely monitored throughout the gastrointestinal tract[11,13]. The dietary lipids activate taste signals in the mouth to promote eating which is stimulated further by other lipid derivatives (*e.g.,* arachidonoylethanolamide or anandamide) synthesized in the gut[9,11,14]. The small intestine also generates anorexic lipid messengers such as oleoylethanolamide (OEA), an endogenous agonist of peroxisome proliferation activator receptors-α (PPARα). The OEA induced activation of PPARα in the intestine leads to stimulation of vagal afferents that innervate key thalamic and striatal nuclei which constantly appraise the sensation of appetite, eating and satiety[15].

The rewarding and re-enforcing aspects of food and whole body energetics are mechanistically linked to dopamine neurotransmission in the midbrain. Chronic consumption of HFD leads to reduced synthesis of OEA thus blunting the nutrient signaling pathway that enables gauging the hedonic value of food; this deficiency causes enhanced craving for HFD and compensatory overeating[13,16]. A preference for fatty foods in rodents can be reversed by supplementation of OEA in their diet[15]. These studies have spurred a systematic search for weight loss agents that may normalize the eating behavior of obese patients and their affinity for obesogenic diets.

Among the many pharmacological and surgical options for the treatment of obesity, none is more effective than RYGB surgery[17-20]. The physiological and neurological underpinnings of RYGB mediated weight loss are only partially understood. However, recent studies make it abundantly clear that massive and sustained weight loss after bariatric surgery involves several mechanisms[20]; these include a key role of the gut microbiome, as was discussed in an earlier FOV Commentary[21].

A prevailing hypothesis to explain eating behavior of obese patients is that they are deficient in perceiving the reward sensation of food. It is further posited that altered striatal dopamine neurotransmission in these individuals underpins their behavior (compensatory overeating) and physiology (higher metabolic set-point). Several recent studies indicate that after undergoing RYGB surgery, both humans and rodents not only eat less but also develop an aversion for fat-enriched foods. Thus, bariatric surgery leads to normalization of

the putative metabolic set-point and food preference; presumably, this occurs by RYGB-induced changes in gut-brain communication[22].

To explore the mechanistic basis of altered dietary preference associated with bariatric surgery, Hankir *et al*[1], studied diet-induced obese rats that underwent RYGB or sham surgery and were exposed to four different experimental regimens. First group of rats underwent sham surgery and was fed regular low fat chow (Sham-LF). The second cohort of rats received sham surgery and was kept on calorically restricted diet; this group served as body weight matched control (Sham-BWM). The third group of rats with RYGB or sham surgery was subject to complete sub diaphragmatic truncal vagotomy (RYGB-VAG and Sham-VAG). Finally, a group of rats with sham surgery was maintained on HFD (Sham-HFD). The authors found that the cohort of Sham surgery rats gained weight on either LFD or HFD. On the other hand, RYGB rats ate less, showed a reduced preference for HFD and lost weight.

To assess if RYGB led to changes in dietary lipid signaling, Hankir *et al*[1], measured the production of OEA in the biliopancreatic limb, the proximal Roux limb or the proximal common channel of RYGB rats. As assessed by liquid chromatography coupled with mass spectrometry (LC-MS), RYGB rats elicited increased OEA synthesis in the most distal areas of their gut. The OEA is known to signal *via* activation of PPARα that relayed these signals *via* vagus nerve afferents to trigger dopamine neurotransmission in the striatum; this signaling pathway, involved in assessing the hedonic value of food, is dampened in obese animals[15]. Consistent with a putative involvement of OEA signaling pathway, the RYGB rats elicited enhanced dopamine neurotransmission in their striatum. The OEA-mediated surge in dopamine signaling pathway was specific to bariatric surgery alone since enhanced dopamine efflux was not seen in sham-operated rats. Similarly, animals that lost weight by caloric restriction did not elicit changes in dopamine neurotransmission nor showed a reduced preference for HFD. The authors also noted that the OEA-mediated signals did not impinge on satiety pathways regulated by oxytocin, as reported previously[23].

Hankir *et al*[1], experimentally assessed if intestinal OEA-sensory vagal afferent-dorsal striatal dopamine signaling pathway was mechanistically linked to lower appetite for fat in RYGB rats. It has been shown earlier that dopamine D2 and D3 receptor levels in dorsal striatum were not affected by RYGB surgery[24-26]. Therefore, Hankir *et al*[1] investigated the expression of striatal D1 receptors (D1Rs) with [^{11}C] SCH-23390 tracer and small animal PET to show that density of D1R was indeed greater in striatum of RYGB rats, regardless of whether they were maintained on LFD or HFD. The authors infused PPARα specific agonist WY-14643 in the gastrointestinal (GI) tract of Sham-LF and Sham-BWM rats to show that pharmacological activation of PPARα triggered a reduced preference for fat and lower intake of HFD in both groups of animals. Conversely, RYGB associated aversion for fat was neutralized by intestinal infusion of a PPARα antagonist (GW-6471). These experiments validated a key role of OEA signaling and its significant alteration by RYGB surgery.

To investigate if OEA-mediating signaling was mechanistically connected to striatal dopamine neurotransmission, authors carried out additional pharmacological interventions. These experiments revealed that infusion of the mixed dopamine receptor antagonist α-flupenthixol in the dorsal striatum neutralized the effect of bariatric surgery on reduced preference for fat in RYGB rats. The eating behavior of RYGB rats was blocked by a D1R selective antagonist (SCH-23390) thus revealing a specific role of D1R in this process. Finally, a simultaneous intestinal infusion of WY-14643 and delivery of SCH-23390 in the striatum cancelled out the effect of activated PPARα signaling. These experimental observations led Hankir *et al*[1] to surmise that OEA mediated gut-brain signaling pathways that determined food preference and satiety were notably re-configured by bariatric surgery. The RYGB specifically led to enhanced intestinal OEA signaling that was relayed by vagus nerve to trigger dopamine neurotransmission in the dorsal striatum. A motivated reader should consult the original paper[22] for its Graphical Abstract summarizing how food-derived signals from the re-configured GI tract following RYGB travel to the reward centers of the brain and lead to reduced preference for fatty foods.

In summary, the observations of Hankir *et al*[1], have shed important light on how anatomical re-configuration of the GI by bariatric surgery leads to profound changes in gut-brain signaling pathways that regulate the motivational and reinforcing aspects of food. These experiments have also revealed that causal links among the OEA-mediated signaling, food preference and weight loss were not absolute since RYGB rats lost considerable amount of weight despite the vagotomy mediated block in the OEA signaling pathway. These data highlight the notion that although dopamine neurotransmission in the ventral striatum is a key sensor of hedonic reward of the food and satiety, these sensations are regulated by mechanisms that utilize additional nutritional and hormonal signals[20,22,26,27]. It is nearly impossible to assess precise contributions of genetic and environmental factors that dictate eating behavior and food preference in humans. The RYGB rat may be an excellent model system to carry out such studies. Finally, we should note that despite its positive clinical attributes, bariatric surgery is by no means risk-free. The RYGB rats represent an ideal model to search for less invasive methods of weight loss in the future.

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REVIEW

Gestational diabetes from A to Z

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Abstract

Gestational diabetes mellitus (GDM) is defined as any degree of hyperglycaemia that is recognized for the first time during pregnancy. This definition includes cases of undiagnosed type 2 diabetes mellitus (T2DM) identified early in pregnancy and true GDM which develops later. GDM constitutes a greater impact on diabetes epidemic as it carries a major risk of developing T2DM to the mother and foetus later in life. In addition, GDM has also been linked with cardiometabolic risk factors such as lipid abnormalities, hypertensive disorders and hyperinsulinemia. These might result in later development of cardiovascular disease and metabolic syndrome. The understanding of the different risk factors, the pathophysiological mechanisms and the genetic factors of GDM, will help us to identify the women at risk, to develop effective preventive measures and to provide adequate management of the disease. Clinical trials have shown that T2DM can be prevented in women with prior GDM, by intensive lifestyle modification and by using pioglitazone and metformin. However, a matter of controversy surrounding both screening and management of GDM continues to emerge, despite several recent well-designed clinical trials tackling these issues. The aim of this manuscript is to critically review GDM in a detailed and comprehensive manner, in order to provide a scientific analysis and updated write-up of different related aspects.

Key words: Diabetes in pregnancy; Diagnostic criteria for gestational diabetes mellitus; Gestational diabetes mellitus-related comorbidities; Genetics of gestational diabetes mellitus; Gestational diabetes mellitus; Lipids abnormalities in gestational diabetes mellitus; Management of gestational diabetes mellitus; Medical nutrition therapy; Pathophysiology of gestational diabetes mellitus; Risk factors for gestational diabetes mellitus

Core tip: Gestational diabetes mellitus (GDM) constitutes a greater impact on the overwhelming diabetes epidemic.

The recent IADPSG revised criteria are considered landmark and evidence based approach in the evolution of screening and diagnosis of GDM. However, there is, still, no consensus on its application, mainly due to concerns related to the benefit of treatment in the additionally diagnosed women and the increased cost. Herein, the authors discuss screening and diagnostic criteria, risk factors, etiology and pathophysiology of GDM along with standard management in antenatal period and during labor.

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INTRODUCTION

From a historical perspective, diabetes in pregnancy was considered as a fatal condition to both mother and foetus prior to the discovery of insulin in 1921[1]. In 1950 Hoet *et al*[2] described the neonatal and obstetric complications of hyperglycaemia in pregnancy. They reported that the "*milieu interieur*" at the time of foetal life was related with the characteristic features of the infant born. According to this theory , this milieu predisposes the child to obesity, hyperglycaemia and ultimately diabetes. Hoet *et al*[2] emphasized on the need to correct the "transitory hyperglycaemia of pregnancy" by insulin, to prevent "meta-gestational diabetes" in mother and metabolic consequences in the infant[2]. Even earlier than Hoet *et al*[2], Jorgen Pedersen reported that the maternal metabolic milieu of hyperglycaemia, increases the foetal blood glucose level and results in pancreatic islet hypertrophy, which in turn increases insulin secretion, consequently increasing also the glucose consumption by the foetus[3]. The Hyperglycemia and Adverse Pregnancy Outcome Study (HAPO), demonstrated that the increase in maternal glucose level was associated with increased umbilical C-peptide and infant's body weight at birth[4].

DEFINITION AND CLASSIFICATION OF DIABETES IN PREGNANCY

The classification of abnormalities of glucose intolerance recognized in pregnancy, is necessary for both epidemiological and clinical purposes. The World Health Organization (WHO) in previous reports defined gestational diabetes mellitus (GDM) as either diabetes or glucose intolerance that is primarily detected during pregnancy. This loose definition of GDM includes a category of "severe hyperglycaemia" lacks a strong evidence basis, from randomized controlled clinical trials (RCTs)[5]. Several trials investigated the association between the glycaemic status of the mother and the outcome in both mother and foetus. However, these trials did not include the category of "severe hyperglycaemia" in their design. For instance, in HAPO study mothers with fasting blood glucose > 5.8 mmol/L and 2-h post oral glucose load > 11.1 mmol/L were excluded[4]. The Australian Carbohydrate Intolerance Study in Pregnant Women (ACHOIS), excluded mothers with fasting blood glucose $\geqslant$ 7.0 mmol/L and 2-h post oral glucose load > 11.0 mmol/L[6]. Moreover, in a study from Landon *et al*[7], mothers with fasting blood glucose $\geqslant$ 5.3 mmol/L were also excluded from the trial. The term "*overt diabetes*" has been used by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) to describe the category of severe hyperglycaemia that was mimicking pre-existing diabetes (PED)[8].

The classic classification system of diabetes in pregnancy was initially developed by Dr. Priscilla White in 1949 and referred currently as the White's classification. On the basis of age at onset, diabetes duration, metabolic, and vascular complications, Dr. White divided diabetes in pregnancy in classes from "A" (more favourable) to "F" (less favourable). The original White' s classification underwent multiple modifications, until 1980[9]. The first revision was done in 1965 by shifting vascular complications to "D" and adding class "R" which denotes the presence of proliferative retinopathy. In 1972 a further update was made in which, GDM was included in class "A" and class "D" was subdivided into five categories. The latest modification applied to the White's classification includes addition of GDM as a distinct separate class and deletion of class "E" and "G"[9]. The American College of Obstetricians and Gynecologists (ACOG) proposed another classification for GDM, adding a note for the presence or absence of metabolic complications, doubting the usefulness of the White's classification in clinical practice[10].

Currently, the term diabetes in pregnancy has been suggested to include all cases of hyperglycaemia observed during pregnancy comprising GDM and PED. The latter include pre-gestational type 2 diabetes mellitus (T2DM) and type 1 diabetes mellitus (T1DM)[11], and GDM is defined as any degree of hyperglycaemia that is recognized for the first time during pregnancy. This definition of GDM should be understood as to include cases of undiagnosed T2DM "*overt diabetes*" identified early in pregnancy and true GDM which develops later in pregnancy[4,7,11] (Figure 1).

UNIVERSAL *VS* SELECTIVE APPROACH OF GDM SCREENING

The screening for GDM, that was established 50 years ago, demonstrates the increased risk of hyperglycaemia during pregnancy[4], and the evidence supporting that effective treatment may reduce hyperglycemia related adverse pregnancy outcomes[6-8]. However, the decision of whether the screening for GDM should be performed

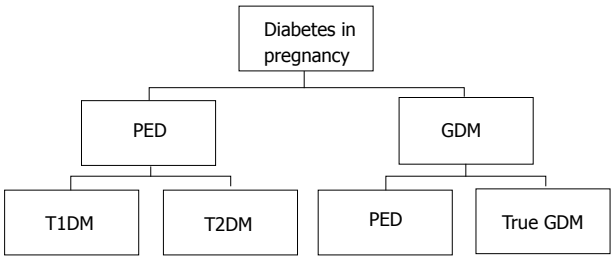

Figure 1 Classification of diabetes in pregnancy[4,7,11]. GDM: Gestational diabetes mellitus; PED: Pre-existing diabetes; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus.

in all pregnant women or selectively in women at high risk of developing T2DM was controversial.

Early screening for GDM is of particular importance, especially in women from population endemic in T2DM. However, early screening for GDM and increased rate of diagnosis is expected to increase psychological stress[11].

In the first antenatal visit, IADPSG recommends either universal or selective screening for women at high risk, to identify women with overt diabetes. In the second phase at 24-28 wk' gestation the IADPSG recommends screening for GDM for all women, *i.e.*, universal screening using 2-h 75g OGTT. Performing selective screening, as recommended by the IADPSG, in early pregnancy is uncontroversial among various expert groups. However, universal screening for all women, using a 75-g OGTT in late pregnancy, remains controversial[8]. The ADA[12] and the ADIPS[13] support universal screening, while the National institute for health and Clinical excellence (NICE)[14] and the Scottish Intercollegiate Guidelines Network (SIGN)[15], recommend selective screening for women with risk factors. Moreover, NICE recommends early screening with a 75-g OGTT in women with previous history of GDM, and at 24-28 wk' gestation for those with risk factors[14].

Current guidelines suggest selective screening during pregnancy based on the presence of risk factors. However, there is no international agreement about which factors will best identify GDM risk, while some of them are defined differently. For instance, BMI > 30 kg/m^2 is suggested by the NICE and SIGN[14,15], as a risk factor for GDM, while ADIPS suggest a BMI > 35 kg/m^2[13], and ADA > 25 kg/m^2[12]. Maternal age was only used by the ADIPS to identify women for selective screening but not by NICE, SIGN or ADA (Table 1)[12-15]. A retrospective observational study designed to assess the selective risk factors recommended by the NICE, ADIPS and ADA was undertaken in women who were screened for GDM using 2-h 75 g OGTT. The sensitivity and specificity of each screening guideline to identify GDM based on these predictors was calculated. The study reported that the most sensitive risk factors for GDM development were the increased maternal age, the increased body weight and past history of GDM. The ADA factors were found to have a sensitivity of 100% and specificity of 3.9%, whereas, both NICE and ADIPS factors had a superior specificity compared to ADA (NICE: 32.4%; ADIPS: 13.7%), but lower sensitivity (NICE: 92.7%; ADIPS: 98.6%). It is obvious that the number of women who would be screened for GDM is higher with the use of the ADA proposed risk factors compared to those proposed by the NICE and the ADIPS, therefor fewer women were exempted from screening, almost similar to the universal screening. On the other hand, less women would be targeted for screening with the use of NICE and ADIPS guidelines, thus, more women with GDM would be missed[16].

Table 1 Risk factors for gestational diabetes mellitus defined by the expert groups[12-15]

NICE[14] and SIGN[15]
BMI > 30 kg/m^2
Previous history of macrosomic baby ⩾ 4.5 kg
Previous history of GDM
Family history of diabetes (first-degree family member with diabetes)
Ethnic backgrounds
South Asian (India, Pakistan or Bangladesh)
Black Caribbean
Middle Eastern (Saudi Arabia, United Arab Emirates, Iraq, Jordan, Syria, Oman, Qatar, Kuwait, Lebanon or Egypt)
ADIPS[13]
Moderate risk factors for GDM
Ethnic backgrounds: Asian, Indian, Aboriginal, Torres Strait Islander, Pacific Islander, Maori, Middle Eastern, and non-White African
BMI 25-35 kg/m^2
High risk factors for GDM
Previous history of GDM
Previous history of high blood glucose
Age ⩾ 40 yr
Family history of diabetes (1st degree relation with diabetes or a sister with GDM)
BMI > 35 kg/m^2
Previous history of macrosomic child ⩾ 4.5 kg
PCOS
Medications: Corticosteroids, antipsychotics
ADA[12]
BMI > 25 kg/m^2
No physical activity
1st degree relation with diabetes
Ethnic backgrounds (African-American, Latino, Native-American, Asian-American, Pacific Islander)
Previous macrosomic child > 9 lb
Previous history of GDM
Hypertension
HDL-C < 0.90 mmol/L and/or triglyceride > 2.82 mmol/L
PCOS
HbA1c ⩾ 5.7% and previous IGT or IFG
Signs of insulin resistance such as acanthosis nigricans
History of CVD

ADA: American Diabetes Association; HDL-C: High-density lipoprotein cholesterol; IFG: Impaired fasting glycaemia; IGT: Impaired glucose tolerance; NICE: National Institute for Health and Clinical Excellence; PCOS: Polycystic ovarian syndrome; SIGN: Scottish Intercollegiate Guidelines Network; ADIPS: Australasian Diabetes in Pregnancy Society; BMI: Body mass index; CVD: Cardiovascular disease; GDM: Gestational diabetes mellitus; HbA1c: Glycosylated haemoglobin.

RISK FACTORS FOR GDM

Common risk factors

Several risk factors have been implicated in the development of GDM. In general, these are similar to

the factors associated with overt diabetes and include increased maternal age, obesity, ethnic background, family history of T2DM and a previous history of GDM. In addition, other risk factors include previous history of a macrocosmic baby, previous adverse pregnancy outcome, glycosuria, polyhydramios or large foetus in present pregnancy[16]. Among these risk factors, increased maternal weight is the most commonly evaluated reversible risk factor. In a nested case-control study, women who presented an increasing weight at a rate of 2.3-10.0 kg/year had a 2.5-times increased risk for GDM[17]. In a population-based study, women with PCOS had 2.4-times increased odds for GDM compared with women without PCOS[18]. Some medications used to treat other conditions, may also affect glucose intolerance increasing the risk for GDM[19,20]. Other reported risk factors include essential hypertension or gestational hypertension and multiple pregnancies[21].

Dietary risk factors

Several cross-sectional and retrospective studies have shown that consumption of macronutrient constituents of the diet during pregnancy may predict development of GDM[21]. Wang *et al*[22] demonstrated an independent significant relationship between reduced intake of polyunsaturated fat and development of GDM. In another study evaluating the effect of lifestyle behavior in white women, revealed a significant correlation of high consumption of saturated fat consumption and risk of GDM, whereas high consumption of polyunsaturated fat was associated with decreased risk for GDM[23]. Moreover, the prospective Nurses' Health Study Ⅱ (NHS-Ⅱ) provided data for several dietary factors among female nurses in the United States in relation to risk for different diseases. In this study the "prudent dietary pattern" included an increased consumption of fruit, green leafy vegetables, poultry, and fish, in contrast to the "Western pattern" which included an increased consumption of red meat, processed meat, refined grain products, sweets, French fries, and pizza. Analyzing the dietary patterns and their association with the risk of GDM development in NHS-Ⅱ trial, it was found a significant relative risk (RR) for GDM development with the increased intake of the Western diet and the decreased intake of prudent diet[24]. Other types of diet that influence the risk of GDM include high glycemic load and a low cereal-fiber diet[21,25].

Micronutrients have also been found to influence glucose tolerance. Zhang *et al*[26] studied the effect of ascorbic acid using data from the prospective OMEGA study. In this study a maternal level of ascorbic acid ⩽ 55.9 μmol/L was found to be associated with a 3.1-times increased risk for GDM compared with maternal level of ⩾ 74.6 μmol/L. Women whose vitamin C intake was < 70 mg per day were found to be associated with a 1.8-times increased risk for GDM compared with higher intakes[26]. The effect of vitamin D status on the risk of GDM was assessed in a nested case-control study from a prospective cohort of pregnant women. A maternal plasma level of 25-hydroxyvitamin D < 20 ng/mL was found in 33% of women diagnosed with GDM compared to 14% in the control group. After adjustment for other confounding factors, a maternal level of 25-hydroxyvitamin D < 20 ng/mL was found to be associated with a 2.66-times increased risk for GDM compared with the control group[27].

SCREENING AND DIAGNOSTIC CRITERIA FOR GDM

From a historical perspective, O'Sullivan *et al*[28,29] first to provide an evidence for the benefits of screening glycaemic abnormalities during pregnancy in women without history of diabetes. They suggested diagnostic criteria for GDM on the basis of a 3-h 100 g oral glucose tolerance test (OGTT) that have been justified to predict later development of diabetes and the risk of increased perinatal morbidity and mortality in pregnant women with GDM[28,29].

A significant debate has been shown to surround the issue of defining glucose abnormalities in pregnancy during the 50 years following the O'Sullivan and Mahan's criteria. The major reason for this dispute in diagnosis is the existence of several diagnostic criteria and glycaemic cut-offs for detection of GDM.

O'Sullivan and Mahan diagnostic criteria for GDM

These were obtained from the results of a study carried out by O'Sullivan *et al*[28] in 1964 and included 752 pregnant women who screened for GDM using 3-h 100 g OGTT. Whole venous blood glucose rather than plasma glucose was estimated using Somogyi-Nelson measurements[30] in four samples (Table 2). Consequently, O'Sullivan *et al*[28,31] was able to predict the development of diabetes over a period of 7-8 years in 29% of women whose whole blood glucose values were more than two standard deviations above the mean. Accordingly, the cut-off values for diagnosis of GDM were estimated based on the mean plus two standard deviations rounded to the nearest 5 mg/dL. These two cut-off values were required to make the diagnosis (Table 2)[28,31].

NDDG criteria

Following the O'Sullivan and Mahan study, glucose levels were measured in plasma rather than whole blood venous samples. Accordingly, the NDDG proposed cut-off values of glucose for GDM diagnosis were the same with the ones proposed by O'Sullivan and Mahan, converted from whole blood to plasma values (Table 2). In NDDG criteria, however, the 1-h blood glucose value changed from 165 mg/dL (O'Sullivan's criteria) to 170 mg/dL without any clarification[32].

Carpenter and Coustan criteria

A further modification was also made to the original O'Sullivan and Mahan diagnostic criteria by Carpenter and Coustan[33] in 1982. This modification was based

Table 2 Various diagnostic criteria for gestational diabetes mellitus and cut-off values

Diagnostic criteria	Sample	WVB (mg/dL)	VP
O'Sullivan and Mahan (Women screened using 3-h 100 g OGTT and two cut-off values are required to diagnose GDM)[28,31]	Fasting	90	90 mg/dL
	1 h	165	165 mg/dL
	2 h	143	145 mg/dL
	3 h	127	125 mg/dL
NDDG criteria (Women screened using 3-h 100 g OGTT and two cut-off values are required to diagnose GDM)[32]	Fasting	90	105 mg/dL
	1 h	170	190 mg/dL
	2 h	145	165 mg/dL
	3 h	125	145 mg/dL
Carpenter and coustan criteria (Women screened using 3-h 100 g OGTT and two cut-off values are required to diagnose GDM)[33]	Fasting	90	95 mg/dL
	1 h	165	180 mg/dL
	2 h	143	155 mg/dL
	3 h	127	140 mg/dL
WHO 1999 criteria (Women screened using 2-h 75 g OGTT and one cut-off value is required to diagnose GDM)[5]	Fasting		126 mg/dL
	2 h		140 mg/dL
Recent IADPSG criteria (GDM) (Women screened using 2-h 75 g OGTT and one cut-off value is required to diagnose GDM)[8]	Fasting		92 mg/dL
	1 h		180 mg/dL
	2 h		153 mg/dL
Recent IADPSG criteria (Overt diabetes) (Women screened using 2-h 75 g OGTT and one cut-off value is required to diagnose GDM)[8]	Fasting		126 mg/dL
	HbA1c		⩾ 6.5%
	RPG		200 mg/dL

GDM: Gestational diabetes mellitus; RPG: Random plasma glucose; VP: Venous blood; WHO: World Health Organization; WVB: Whole venous blood; HbA1c: Glycosylated haemoglobin; IADPSG: International Association of Diabetes and Pregnancy Study Groups; NDDG: National Diabetes Data Group; OGTT: Oral Glucose Tolerance Test.

on the fact that the whole blood glucose determined by the non-specific Somogyi-Nelson technique measures both glucose and other reducing constituents. In the late 1970s, glucose levels were measured using glucose oxidase technique. With this method glucose levels were approximately 5 mg/dL lower compared to Somogyi-Nelson technique[34]. Accordingly, Carpenter and Coustan[33] used the original O'Sullivan and Mahan values by subtracting 5 mg/dL from the blood glucose values to offset the difference in the analytic method used, and added 14% to offset the variation of changing from whole blood to plasma values[30,34] (Table 2).

The glucose cut-off values in the Carpenter and Coustan criteria were lower than that in O'Sullivan and the NDDG diagnostic criteria. This may explain, in part, the increasing prevalence of GDM in the years followed. Ferrara *et al*[35] demonstrated this theory in a cohort of multi-ethnic Northern California women who were not known to have diabetes before. The population was screened for GDM using 1-h 50 g OGTT and those who had a plasma glucose ⩾ 140 mg/dL underwent further 3-h 100 g OGTT. In this cohort the prevalence of GDM appeared to be significantly increased when applying Carpenter and Coustan criteria compared to NDDG criteria[35].

WHO 1999 criteria

The WHO criteria include a 2-h 75 g OGTT test. This test was first introduced in the 1980s for type 2 diabetes and glucose abnormalities diagnosis. In particular, a 75 g of glucose were administrated orally to a pregnant woman following by an overnight fast of 8 to 14 h. Plasma glucose was measured in the fasting state, and 2 h later. Unlike the 3-h 100 g OGTT, 1 h and 3 h measurements were not required. Only one cut point was sufficient to diagnose GDM (Table 2)[5]. Compared to the O'Sullivan and Mahan diagnostic criteria, the WHO 1999, criteria were not evidence-based, as their cut-off values were selected arbitrary according to expert opinion and consensus. However, the validity of this test as screening tool was only evidenced recently after being used in the HAPO study[4].

Recent IADPSG criteria

In 1998, IADPSG organization was established. The main goal of this organization was to enable cooperation between different national and international societies with principal interest on diabetes in pregnancy. The evidence provided by the HAPO study, formed the basis of the recent IADPSG criteria for screening and diagnosis of GDM[8]. HAPO study included 25505 pregnant women from a diverse, heterogeneous, multinational population from 15 centers, designed to evaluate the risk of adverse outcomes related to the maternal glycaemic values that previously were considered normal. GDM was screened with a 2-h 75 g OGTT at 24- to 32-wk gestation[4]. Unlike previous studies, HAPO study was designed to evaluate the development of adverse pregnancy outcomes rather than future development of T2DM[4]. In 2008 the IADPSG consensus panel decided to set the level of glucose thresholds for GDM diagnosis based on the odds ratio of 1.75 relative to the mean for specific adverse outcomes. Accordingly, the glucose thresholds for diagnosis of GDM were calculated as the average glucose levels at which odds ratios for adverse outcomes have attained 1.75 times the estimated odds ratios for their development. The Consensus Panel members of the IADPSG also reviewed the data

surrounding the issue of overt diabetes detected during pregnancy. Due to lack of evidence from well-designed RCTs they proposed diagnostic criteria based on expert opinion. The IADPSG recommended screening for the presence of this category during the first antenatal visit by performing FPG, random plasma glucose (RPG) or glycosylated haemoglobin (HbA1c). Only one abnormal value was proposed to be sufficient to diagnose overt diabetes (Table 2). A 2-h 75 g OGTT earlier than 24-28 wk of gestation was not routinely advised. However, it was proposed that all pregnant women should undertake this test at 24-28 wk of pregnancy excluding those with diagnostic results for overt diabetes or GDM at first antenatal visit. Upon performing 2-h 75 g OGTT at 24-28 wk gestation, GDM is recognized based on the cut-off values of FPG, 1-h plasma glucose, or 2-h plasma glucose (Table 2). It is noteworthy that, only one abnormal glucose value is sufficient to diagnose GDM, while a FPG value of more than 92 mg/dL in early pregnancy is sufficient to diagnose GDM[8].

Falavigna *et al*[36] reported significant benefits of screening and subsequent treatment of GDM with the use of IADPSG criteria compared to the previous WHO 1999 criteria. Consequently, the 2011 ADA guidelines recommended IADPSG criteria for screening and diagnosis of GDM[37]. However, three years later (2014) ADA guidelines, recommended either the "one-step" approach performed using 2-h 75-g OGTT, or the previous Carpenter and Coustan "two-step" approach using 1-h 50-g OGTT screening followed by 3-h 100-g OGTT[12]. The reason behind this change in ADA guidelines was addressed by the National Institutes of Health (NIH) as a reason for the increasing prevalence of GDM noting also the uncertainty related to benefit of treatment in the additionally diagnosed women as a result of the new criteria[38].

Cost-effectiveness of detecting GDM using the new criteria

Obviously the increased prevalence of GDM upon using the Carpenter and Coustan criteria would lead to greater health care cost. Studies demonstrated that women with GDM diagnosed by the Carpenter and Coustan criteria but not identified by the NDDG criteria had increased risk of adverse outcomes such as macrosomia, neonatal hypoglycaemia, and hyperbilirubinemia[35,39].

The IADPSG criteria have been adopted by various expert groups including the ADA[40], the WHO[5] and the Australasian Diabetes in Pregnancy Society (ADIPS)[13]. However, the concerns related to the benefit of treatment in the additionally diagnosed women and the increased cost of the health care services impedes its wide use. The recently conducted prospective St. Carlos Gestational Diabetes Study was designed to assess the cost-effectiveness of the "one-step" IADPSG, compared with the "two-step" Carpenter and Coustan criteria. The prevalence of GDM in the population evaluated by Carpenter and Coustan criteria was 10.6% and reached 35.5% when using the IADPSG criteria. This study demonstrated a reduction in the rate of gestational hypertension, prematurity, need for cesarean delivery, small for gestational age (SGA), Large for Gestational Age (LGA) and admission to Neonatal Intensive Care Unit (NICU) with the use of the new criteria. In addition, a total of €14358.06 per hundred women would be saved if IADPSG criteria were applied instead of Carpenter and Coustan criteria[41]. Therefore this recent evidence demonstrates improved pregnancy outcomes together with cost-effectiveness despite the rise in prevalence which may supports the use of the IADPSG criteria as an international standard approach.

EPIDEMIOLOGY

Epidemiology data are useful in both health care planning and cost savings. The prevalence of GDM has been progressively increasing and it reflects the background prevalence of obesity and T2DM in general population[42,43]. Higher rates of GDM were found to raise in parallel with higher rates of T2DM. This may be related to the common risk factors including obesity, physical inactivity, ethnic background and urbanization[44]. In a cohort of 123040 Northern Californian pregnant women without pre-existing diabetes, Hedderson *et al*[45] reported that the prevalence of GDM was low among non-Hispanic white women and African Americans, and high in Asians and Filipinas (Figure 2). Interestingly higher rates of GDM were demonstrated among those with lowest BMI (Asians and Filipinas) and lower rates were found in those with highest BMI (non-Hispanic white women and African Americans)[45].

The exact prevalence rate of GDM remains unknown and may differ widely based on the diagnostic criteria used for screening. In recent years the diagnostic criteria have changed considerably and there has been no agreement about which criteria to use. In the new IADPSG criteria only one value is sufficient to confirm the diagnosis and this may increase the prevalence of GDM to rates as high as 15%-20%[8].

Variation in prevalence rates could also be related to diversity of the populations being studied. In populations at low risk for GDM, such as in Sweden, the prevalence is less than 2%, while in those at high risk, such as the Indigenous American, Northern Californian Hispanics and Northern Californian Asians, the prevalence ranges from 4.9% to 12.8%[46]. Also higher rates were observed in Middle East countries such as in United Arab Emirates (20.6%), Qatar (16.3%), Bahrain (13.5%) and Saudi Arabia (12.5%). Some developed countries have also higher prevalence rates such as Canada (17.8%) and France (12.1%), but lower rates were observed in Australia (9.5%) and 4.8% in United States[46].

ETIOLOGY AND PATHOPHYSIOLOGY OF GDM

Pregnancy represents a complex metabolic and physiological condition that can be considered as a

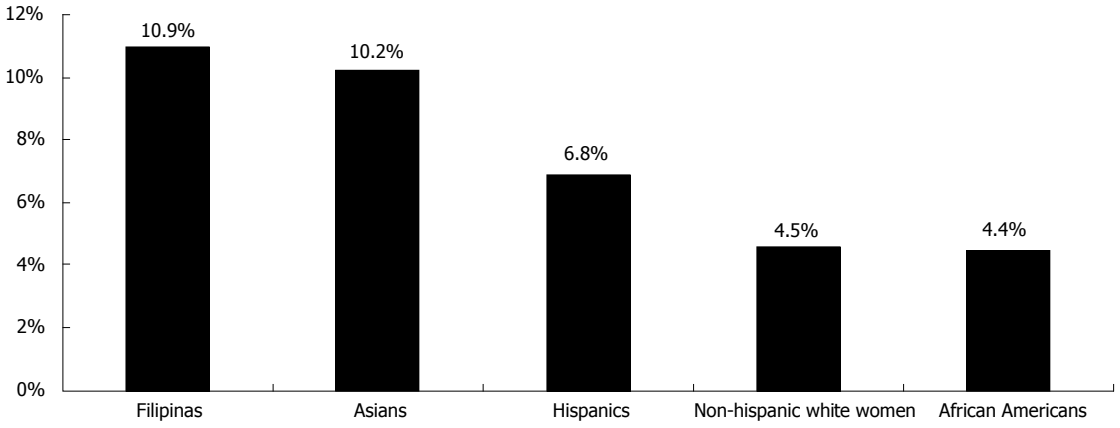

Figure 2 Ethnic variations in the prevalence of gestational diabetes mellitus[45].

status of biological tolerance test which has the ability to detect insulin resistance earlier[11]. Insulin resistance in pregnancy could be the result of maternal obesity with varying degree of adipocytokine production, or increased production of diabetogenic placental hormones. In addition to insulin resistance, pancreatic β-cell dysfunction might also play a role in the pathophysiology of GDM (Table 3).

Obesity, pregnancy and inflammatory status

Obesity and overweight are nearly frequent findings among women in their childbearing years. In the United Kingdom 32% of women whose age ranges between 35-64 years old are overweight and 21% of them are obese[47]. Obesity is considered a state of chronic inflammation in which inflammatory markers are produced in excess to systemic circulation. These inflammatory markers influence alterations in post-receptor insulin signaling resulting in increased insulin resistance[48]. Moreover, pregnancy *per se* is an additional inflammatory condition in which there is physiological adaptation of the innate immune system to prevent rejection of the growing foetus. In normal pregnancy, the cytokines produced by humoral immunity significantly predominate in activity over that produced by cell-mediated immunity. This strong shifting towards humoral immunity has a beneficial role in maintaining a good relationship between mother and foetus at the expense of creating an inflammatory milieu that increases insulin resistance[49].

New potential mediators of insulin resistance

The adipose tissue is an endocrine organ that produces adipocytokines including pro- and anti-inflammatory mediators such as leptin, adiponectin, and resistin. Obesity is associated with an alteration in adipocytokines production from both adipocytes and macrophages. These inflammatory mediators may act locally to aggravate inflammation in adipose tissue, and increases peripheral insulin resistance. Altered adipocytokines production can also act centrally on the hypothalamus, promoting increased food intake and hyperglycemia. During pregnancy, adipocytokines have been shown to influence glucose tolerance *via* mechanisms interfering with regulation of insulin secretion and insulin receptor signaling and it explains, in part, the development of insulin resistance[50].

Adiponectin is an adipocytokine polypeptide that has anti-inflammatory properties and insulin sensitizing action. Williams *et al*[51] reported that a maternal level of adiponectin < 6.4 μg/mL was associated with a 4.6-times increased risk for GDM compared with control group. Ranheim *et al*[52] has also demonstrated reduced adiponectin mRNA levels in biopsies from abdominal subcutaneous adipose tissues and reduced plasma level in women with GDM, independently of obesity. A meta-analysis of 25 prospective studies reported that a level of adiponectin < 2.25 μg/mL in early pregnancy, was associated with significant risk for GDM compared with normal pregnant women[53].

Tumor necrosis factor-α (TNF-α) interferes with insulin receptor signaling and β-cell function having a greater influence in hyperglycaemia[50,54]. Three observational studies and one meta-analysis found that women with GDM had significantly higher levels of TNF-α compared with normal glycaemic pregnant women[55-58]. However, conflicting results have been reported in other studies[59-61]. This could be related to the smaller population sizes and the diversity in matching and adjustment for the confounding variables.

Interleukin-6 (IL-6); an inflammatory marker that has been found to be significantly higher in women with GDM, compared with normal women, independent of adiposity[56,62,63]. Similarly, a recent cross-sectional trial showed that IL-6 could be independently used to predict development of GDM when assessed in the first trimester[64].

Leptin is a protein hormone related to the bulk of fat stores[54] that has been reported significantly elevated in women with GDM compared with controls[56,58,65,66]. In a predictive risk model it was proposed that each 10 ng/mL increase of leptin levels, was associated with a 20% increase risk for GDM[66]. However, some trials reported conflicting results[67,68].

Table 3 Etiology and pathophysiology of gestational diabetes mellitus

Insulin resistance
Pregnancy and obesity as states of low grade inflammation[48,49]
Adipocytokines
↓ Adiponectin[51-53]
↑ TNF-α[55-58]
↑ IL-6[56,62-64]
↑ Leptin[56,58,65,66]
↑ AFABP[79]
↑ RBP-4[69]
?↑ Resistin[54,62,72]
?↑ Visfatin[76,77]
? Novel adipocytokines (Vaspin, Apelin and Omentin)[50]
Endothelial function and angiogenic growth factors[74,80,81]
↓ EPC
↓ SOD
↑ eNOS
↑ PAI-1
↑ sEng
↑ sICAM-1
↑ sVCAM-1
↑ t-PA
↑ PLGF
↑ sFlt-1
Proteomics biomarkers
Haptoglobin, protein SMG8 and apoptosis inducing factor-1[83]
Apolipoprotein CIII[84]
Peptides precursors of clusterin, isoform 1 of fibrinogen alpha chain and apolipoprotein CII[85]
Glycosylated fibronectin[86]
Transthyretin–retinol binding protein-retinol complex[87]
Pancreatic β-cell dysfunction
Autoimmunity[92]
Glucokinase gene defect[93]

AFABP: Adipocyte fatty acid-binding protein; eNOS: Endothelial nitric oxide synthase; sEng: Soluble endoglin; sFlt-1: Soluble fms-like tyrosine kinase-1; sICAM-1: Soluble intercellular adhesion molecule-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; SOD: Superoxide dismutase; t-PA: Tissue plasminogen activator; TNF-α: Tumor necrosis factor-α; RBP-4: Retinol-binding protein-4; EPC: Endothelial progenitor cells; hPGH: Human placental growth hormone; hPL: Human placental lactogen; IL-6: Interleukin-6; PLGF: Placental growth factor; PAI-1: Plasminogen Activator Inhibitor type-1.

Retinol-binding protein-4 (RBP-4) a carrier for retinol (vitamin A) that has been demonstrated significantly increased in women with GDM compared with controls[69], while other studies found no significant differences[70,71]. These conflicting results may be related to the design of the studies, which were mostly cross-sectional, limiting their ability to detect a causal relationship.

Resistin is a peptide hormone related to energy homeostasis and has been shown to be elevated in women with GDM compared to normal pregnancies[54,62,72]. However, Megia *et al*[73] reported lower resistin levels in women with GDM compared with euglycaemic women while other studies reported no difference[74,75].

Visfatin is a protein related to glucose homeostasis that has been demonstrated to be significantly higher in women with GDM compared with controls[76,77]. However Chan *et al*[78] reported decreased serum visfatin in Chinese women with GDM.

Adipocyte Fatty Acid-Binding Protein (AFABP) has been shown to be significantly elevated in a cross-sectional study in women with GDM compared with controls[79].

Vaspin, apelin and omentin are novel adipokines with a controversial role in the pathogenesis of GDM. Some studies demonstrated increased levels, while others report unchanged or even decreased levels. Most of these trials were cross-sectional in design. The lack of controlled prospective trials are limiting further conclusions on their role on GDM[50].

Endothelial functions and angiogenic growth factors

Several studies have shown that endothelial function and angiogenic growth factors were altered in women with GDM. For instance, a case-control study demonstrated decreased total endothelial progenitor cells (EPC), decreased expression of superoxide dismutase (SOD), increased levels of soluble adhesion molecules (both sICAM-1and sVCAM-1) and increased endothelial nitric oxide synthase (eNOS) expression in women with GDM compared with controls. These alterations in endothelial function were also present in fetuses of GDM mothers which might infer an increased risk of future development of T2DM and CVD[80]. In one cross-sectional study, tissue plasminogen activator (t-PA) (reflect endothelial activation) was found significantly elevated in women with GDM[81]. Increased levels of Plasminogen Activator Inhibitor type-1 (PAI-1) have been also reported to be associated with increasing glucose levels in a subgroup of pregnant women from the HAPO study[74].

Proteomics and metabolomics for early prediction of GDM

Proteomics is a wide-ranging analysis of samples produced in humans using mass spectrometry, capable of generating huge data about proteins produced. In a recent review by Singh *et al*[82], several technologies were developed to identify protein biomarkers that serve as early predictors of GDM development. Wang *et al*[83] used Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS PAGE) and mass spectrometry (MS) tool to separate proteins from sera of women with GDM and hypertensive disorder. They identified haptoglobin, protein SMG8 and apoptosis inducing factor-1 as potential markers for GDM development. Kim *et al*[84] used surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) and MS to analyze serum samples from healthy women at 16-20 wk gestation. They demonstrated that apolipoprotein CIII was significantly higher in women who later developed GDM compared with controls, but there was no difference between the two groups when apolipoprotein AII level was investigated. Ai *et al*[85] used Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) followed by weak cation exchange magnetic bead (WCX-MB) to identify lower molecular weight peptides in healthy women at

their 20 wk of gestation. Subsequently, they showed that three peptides were significantly different in women who later developed GDM compared with controls. These three peptides were demonstrated to be precursors of clusterin, isoform 1 of fibrinogen alpha chain and apolipoprotein CII[85]. Similarly, other studies using these novel techniques, have shown that glycosylated fibronectin[86] and the transthyretin-retinol binding protein-retinol complex[87] were early predictors of GDM, even before the onset of glucose intolerance.

Diabetogenic placental hormones

Ryan *et al*[88] provided evidence for the role of placental hormones in the induction of insulin resistance in pregnant rats. They suggested that increasing levels of progesterone, cortisol, prolactin and human placental lactogen (hPL) play a causal role in the insulin resistance during pregnancy, but whether they have the same effect in human pregnancy remains to be elucidated. hPL has been suggested to be a major contributory factor of insulin resistance in humans[89].

Pancreatic β-cell dysfunction

Another etiology for GDM was thought to be related to β-cell dysfunction that occurs on the setting of insulin resistance state[90]. Xiang *et al*[91] have shown a reduction of pancreatic β-cell function by 67% in women with GDM compared with normal glucose tolerance controls. This impairment of β-cell function was thought to be attributed to an autoimmune process[92]. However, Molęda *et al*[93] excluded autoimmunity as a cause of GDM based on negative anti-glutamic acid decarboxylase (anti-GAD) antibodies test and suggested a genetic defect which might be responsible for the β-cell secretory dysfunction.

GENETICS OF GDM

Studies demonstrated increased risk of GDM in women with family history of T2DM compared with those of matched controls[94]. Insulin resistance and insulin insufficiency were characteristic features of GDM, and both were partly shown hereditary in a recent study in twins[95].

GDM could be considered a polygenic, heterogeneous disease similar to T2DM in which multiple factors act together to cause the condition. The genes studied in relation to GDM are categorized as those related to insulin secretion, insulin and insulin receptors, insulin resistance and energy metabolism, human leukocyte antigen (HLA) and others (Table 4).

Genes related to insulin secretion

***KCNJ11* and *ABCC8* genes:** Pancreatic β-cell insulin secretion is dependent on the functional integrity of the K-ATP channel which is composed of eight subunits; four Kir6.2 subunits encoded by potassium channel inwardly rectifying subfamily J member 11 (*KCNJ11*) and four sulfonylurea receptor-1 (SUR1) subunits encoded by ATP-binding cassette transporter sub-family C member 8 (*ABCC8*)[96]. One study demonstrated that two variants of the *ABCC8* gene; the tagGCC allele of exon 16 and the AGG allele of the R1273R were significantly associated with GDM compared with controls[97]. In a large case-control study, Shaat *et al*[98] reported a significant association between E23K genetic variant within the *KCNJ11* gene and GDM compared with controls.

Uncoupling protein-2 gene: Uncoupling protein-2 *(UCP-2)* is a trans-membrane mitochondrial carrier protein which facilitates mitochondrial proton leak, thereby reducing ATP production resulting in inhibition of insulin secretion[99]. In a large case-control study, Shaat *et al*[98] showed no significant difference in the rate of the UCP2-866G>A variant within the *UCP-2* gene in Scandinavian women with GDM compared with controls.

Mitochondrial NADH dehydrogenase-1 (*MT-ND1*) gene: In a case-control study, Chen *et al*[100] showed that the T3398C mutation within the *MT-ND1* gene was significantly associated with GDM compared with normoglycaemic controls. This mutation may alter the function of NADH dehydrogenase resulting in impairment of the mitochondrial ETC with a subsequent reduction in insulin secretion.

Transcription factor 7-like2 (*TCF7L2*) gene: Two studies showed a significant association between the T allele of the rs7903146 genetic variant within the *TCF7L2* gene and GDM compared with non-diabetic controls[101,102].

Glucokinase (GCK) (MODY2) gene: Two studies found no significant association between the rs1799884 (-30G/A) variant and risk of GDM[103,104]. However, two other recent large studies demonstrated a significant association[105,106]. Moreover, a meta-analysis including seven studies, also demonstrated a significant association between the rs1799884 variant and risk of GDM[107].

Hepatocyte nuclear factor 4 alpha (*HNF4A, MODY1*) gene: Three variants within the *HNF4A, MODY1* gene (rs2144908, rs2425637 and rs1885088) were tested in a case-control study and were found not to be associated with risk of GDM[105].

Hepatocyte nuclear factor 1 alpha (*HNF1A, MODY3*) gene: Two variants within the *(HNF1A, MODY3)* gene were examined in two case-control studies. Shaat *et al*[105] found an association of the rs1169288 (Ile27Leu) variant and risk of GDM that was not statistically significant whereas Lauenborg *et al*[108] showed no association of the *rs*1800574 (Ala98Val) variant with GDM.

Genes of insulin and insulin receptors

Insulin (*INS*) gene: This gene contains a promoter

Table 4 Genetic variants studied in relation to gestational diabetes mellitus

Gene	Location	Variant	Association
Genes related to insulin secretion			
ABCC8	11p15.1	tagGCC allele of exon 16 and the AGG allele of the R1273R	Significant[97]
KCNJ11	11p15.1	E23K	Significant[98]
UCP-2	11q13	UCP2-866G> A	Controversial[98]
MT-ND1	mtDNA	T3398C mutation	Significant[100]
TCF7L2	10q25.3	rs7903146	Significant[101,102]
GCK	7p15.3-p15.1	rs1799884 (-30G/A)	Significant[105-107]
HNF4A	20q13.12	rs2144908, rs2425637 and rs1885088	No association[105]
HNF1A	12q24.2	rs1169288, rs1800574	No association[105,108]
Genes of insulin and insulin receptors			
INS	11p15.5	INS-VNTR class-III allele	Controversial[110,111]
INSR	19p13.3-p13.2	INSR allele-1 Kpn I RFLP	Significant[112]
IGF2	11p15.5	IGF2 Bam HI RFLP	Significant[112]
IGF2BP2	3q27.2	rs4402960	Significant[113-115]
IRS1	2q36	rs1801278 (Gly972Arg)	Controversial[98,107]
Genes of insulin resistance			
PPARG	3p25	rs1801282	No association[107]
PPARGC1A	4p15.1	rs8192678	No association[101,118]
ADRB3	8p11.23	rs4994 (Trp64Arg)	Controversial[101,194,119-121]
SLC2A1	1p34.2	SLC2A1 Xba I RFLP	No association[112]
ADIPOQ	3q27	rs1501299	No association[101]
FOXC2	16q24.1	-512C allele	No association[101]
HLA genes			
HLA	6p21	DR3 and DR4	Controversial[122,123,125]
HLA	6p21	DR3-DQ2/X, DR4-DQ8/X with positive autoantibodies	Associated[124]
HLA	6p21	DR7-DQ2/X, DR9-DQ9/X and DR14-DQ5/X	Associated[124]
HLA	6p21	DQB1 alleles	Associated[111]
Other genes			
CAPN10	2q37.3	SNP-19, SNP-43, SNP-44, SNP-63)	No association[98,101]
HFE	6p21.3	C282Y in Northern and Central European women	Associated[127]
HFE	6p21.3	H63D	No association[127]
MBL2	10q11.2	rs1800450 (Gly54Asp)	Significant[128]
MBL2	10q11.2	rs5030737 (Arg52Cys)	No association[128]
SERPINE1	7q22.1	-675 4G/5G	Could be associated[130]

ABCC8: ATP-binding cassette transporter sub-family C member 8; ADIPOQ: Adiponectin ADRB3 adrenergic receptor β3; CAPN10: Calpaine 10; FOXC2: Forkhead box C2; GCK: Glucokinase; HFE: Haemochromatosis; HLA: Human leukocyte antigen; HNF4A: Hepatocyte nuclear factor 4 alpha; HNF1A: Hepatocyte nuclear factor 1 alpha; IGF2BP2: Insulin-like growth factor-2 mRNA-binding protein-2; IGF2: Insulin-like growth factor 2; IRS1: Insulin receptor substrate 1; INS: Insulin; INSR: Insulin receptor; KCNJ11: Potassium channel inwardly rectifying subfamily J member 11; MBL2: Mannose binding lectin 2; MT-ND1: Mitochondrial NADH dehydrogenase-1; PPARG: Peroxisome proliferator-activated receptor gamma; PPARGCIA: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RFLP: Restriction fragment length polymorphism; SERPINE1: Serpin peptidase inhibitor, clade E, member 1; SLC2A1: Solute carrier family 2 (facilitated glucose transporter), member 1; SNP: Single nucleotide polymorphism; TCF7L2: Transcription factor 7-like 2; UCP-2: Uncoupling protein-2; VNTR: Variable number of tandem repeats.

region of variable number of tandem repeats (VNTR) with VNTR class-Ⅰ allele occurs more frequently than VNTR class-Ⅲ allele[109]. Litou *et al*[110] reported a significant association between the rate of VNTR class-Ⅲ allele and risk for GDM. However Shaat *et al*[111] failed to demonstrate such an association.

Insulin receptor (*INSR*) gene: *INSR* gene restriction fragment length polymorphisms (RFLPs) was tested by Ober *et al*[112] and showed that, the *INSR* allele-1 Kpn I RFLP was significantly associated with GDM among black and Caucasian women. Interestingly, the influence of BMI on the risk of GDM was found to be significant only in individuals with positive *INSR* allele-1, which would suggest a possible role of the *INSR* allele-1 in obesity[112].

Insulin-like growth factor-2 (*IGF2*) gene: Ober *et al*[112] showed an increased risk of GDM in Caucasian women who are carriers of *IGF2* Bam HI RFLP, but only in the presence of *INSR* allele-1.

Insulin-like growth factor-2 mRNA-binding protein-2 (*IGF2BP2*) gene: In a recent large study, Cho *et al*[113] showed a significant association between rs4402960; a genetic variant of the *IGF2BP2* gene and risk of GDM in Korean women. Similarly, Wang *et al*[114] and Lauenborg *et al*[115] showed the same results among Chinese and Danish Caucasian women respectively.

Insulin receptor substrate 1 (*IRS1*) gene: Shaat *et al*[98] failed to demonstrate a significant association between the T-allele of the rs1801278 (Gly972Arg) variant and risk of GDM. However, in a meta-analysis of four studies, a significant association was demonstrated[107].

Genes of insulin resistance and energy metabolism

Peroxisome proliferator-activated receptor gamma (*PPARG*) gene: *PPARG* gene encodes production of a factor that is essential in the regulation of adipocytes, the differentiation and metabolism of lipids and glucose[116]. A meta-analysis of eight studies showed no association of *PPARG*/rs1801282 variant and risk for GDM compared with controls[107].

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) gene: *PPARGC1A* gene encodes production of a protein that plays a role in regulation of genes related to energy metabolic processes[117]. Two case-control studies among Scandinavian and Caucasian women demonstrated that the rs8192678 variant of the *PPARGC1A* gene is not associated with GDM[101,118].

Adrenergic receptor β3 (*ADRB3*) gene: One study showed a significant association between the rs4994 variant of the (*ADRB3*) gene and the risk of developing GDM among Caucasian women[119]. However, other studies failed to confirm this association among Scandinavian, Caucasian and Taiwanese women[101,120,121]. Similarly a meta-analysis of five studies also showed no association between the rs4994 variant and risk of GDM[107].

Solute carrier family 2 (facilitated glucose transporter), member 1 (*SLC2A1*) gene: Ober *et al*[112] failed to demonstrate an association between two fragments of the *SLC2A1* gene Xba I RFLP and risk of GDM.

Adiponectin (*ADIPOQ*) gene and Forkhead box C2 (*FOXC2*) gene: Shaat *et al*[101] tested the rs1501299, a variant within *ADIPOQ* gene and -512C allele variant within *FOXC2* gene in a large population of Scandinavian women and found no differences in variant rate among women with GDM and controls.

HLA genes

Freinkel *et al*[122] reported a significantly higher rate of *HLA-DR3* and *HLA-DR4* among black women with GDM. Similarly, Ferber *et al*[123] showed a significant association of the *HLA-DR3* allele among GDM women who were positive to islet cell autoantibodies (ICA), and *HLA-DR4* allele in those who were positive to glutamic acid decarboxylase autoantibodies (GAD65Ab). Another Swedish study showed increased frequency of *HLA-DR3-DQ2/X* or *DR4-DQ8/X* among women with GDM and positive autoantibodies, and *HLA-DR7-DQ2/X, DR9-DQ9/X* and *DR14-DQ5/X* among women with GDM and negative autoantibodies[124]. Moreover, Shaat *et al*[111] showed that *HLA-DQB1* alleles were associated with increased risk of GDM among Scandinavian women. However Rubinstein *et al*[125] found no differences in variant rates of *HLA-DR3* and *HLA-DR4* among women with GDM and controls.

Other genes

Calpaine 10 gene: Two studies had tested the single nucleotide polymorphisms (SNPs) within the calpaine 10 (*CAPN10*) gene. Shaat *et al*[98] found no difference between the rate of SNP-43 and SNP-44 among women with GDM and controls and similar results were obtained by Leipold *et al*[126] when they tested SNP-43, SNP-19 and SNP-63 variants.

Haemochromatosis gene

In a case-control study, Cauza *et al*[127] tested both C282Y and H63D variants within haemochromatosis (*HFE*) gene in a large population of women. They showed an increased rate of C282Y but not H63D variant among Northern and Central European women with GDM compared with controls. However the rate of both variants did not differ between Southern and non-European women with GDM and controls[127].

Mannose-binding lectin 2 gene: The rate of Arg52 Cys variant within the mannose-binding lectin 2 (*MBL2*) gene in women with GDM does not differ from that in controls. However, the Gly54Asp variant was found significantly associated with increased risk of GDM compared with controls[128].

Serpin peptidase inhibitor, clade E, member 1 (*SERPINE1*) gene: The most common allele is in the promoter region at position -675 and has a run of 5 G's. The 4/G allele can result from deletion of one nucleotide and accordingly (4G/4G, 4G/5G, and 5G/5G) genotypes are recognized. The 5G/5G genotype is associated with decreased levels of PAI-1 activity[129]. Leipold *et al*[130] demonstrated a significantly higher rate of the 5G/5G genotype among women with normal fasting and glucose tolerance which may suggest a possible role of the -675 4G/5G variant in the increased risk of GDM.

ABNORMALITIES AND SIGNIFICANCE OF LIPIDS IN GDM

Brizzi *et al*[131] showed a significant increase in serum triglycerides, total cholesterol and LDL-C during normal pregnancy, with HDL-C level only slightly decreased. Metzger *et al*[132] showed an increase in serum triglyceride and plasma free fatty acids during the third trimester of pregnancy. However an elevation in serum cholesterol level did not show a significant difference. A meta-analysis of 60 studies showed significant elevation of triglycerides, non-HDL-C and decreased HDL-C level in women with GDM compared with control. Again, no difference was found in cholesterol or LDL-C levels between the two groups[133].

In addition, lipid abnormalities may also affect foetal growth similar to that of elevated plasma glucose. Kitajima *et al*[134] reported a significant association between maternal triglyceride levels at mid-term and

the risk of foetal macrosomia. In a recent study, Son *et al*[135] related higher risk of developing large for gestational age (LGA) newborn to increased levels of maternal triglyceride. Moreover, in a prospective study of 150 pregnant women, maternal triglycerides and FFA levels were independent factors associated with LGA[136].

Lipid abnormalities might serve as predictors of later GDM development. In a cross-sectional study, Savvidou *et al*[81] showed that a decreased level of HDL-C at first trimester is predictive of later development of GDM.

GDM-RELATED COMORBIDITIES

Several studies demonstrated a clear association between maternal glucose levels and adverse consequences to both mother and foetus, and this continuous relationship has been also shown related to mild glucose levels[4,137]. These adverse consequences are independent of other factors such as BMI and increasing weight during pregnancy as shown in the HAPO study[4].

Maternal comorbidities

Hypertensive disorders: The reason behind development of hypertension in patients with diabetes is attributed to the effect of hyperinsulinemia on increasing weight, and renal sodium retention[138]. Hypertensive disorders during pregnancy are classified into three categories; chronic hypertension ,preeclampsia and gestational hypertension[139]. The HAPO study demonstrated that GDM women with the highest BMI had eight times the likelihood of developing preeclampsia as women with the lowest BMI[4]. Barden *et al*[140] showed increased risk of preeclampsia in women with GDM compared with controls. On the contrary, in a recent retrospective study, GDM and chronic hypertension were found protective against development of preeclampsia and gestational hypertension[141]. In the long-term hypertensive disorders carry the risk of developing T2DM, hypertension, metabolic syndrome and CVD[142].

Preterm birth: This is defined as infants born alive prior to 37 wk gestation[4,7]. In the HAPO study 6.9% of the total participants had preterm birth, but less frequently encountered than neonatal requirement for NICU (8.0%) and birth weight > 90th percentile (9.6%). Preterm birth was shown significantly associated with increased post OGTT maternal glucose but not fasting glucose levels[4].

Shoulder dystocia: Traumatic vaginal delivery resulting from delivery of large babies exposes the woman with GDM to operative procedures and episiotomies[143]. Shoulder dystocia may also occurs in infants weighing < 4.0 kg[138] and necessitates the use of operative procedures to deliver the shoulders[144]. In the HAPO study shoulder dystocia was less frequently encountered compared with other outcomes (1.3%) and was shown associated with increased post OGTT maternal glucose and also fasting glucose levels[4].

Caesarean delivery: Usually is required to overcome an adverse complication associated with GDM such as shoulder dystocia, and since it is a major surgical procedure, it carries the risk of complications such as infection, bleeding, thrombosis and wound dehiscence[144]. In the HAPO study 16.0% of the total participants had a primary Caesarean delivery and 7.7% had a repeated Caesarean delivery and both had been shown associated with increased post OGTT maternal glucose and fasting glucose levels[4]. The Toronto Trihospital study showed that Caesarean deliveries were still conducted despite reduced infant's birth weights in treated women with GDM[39] which might suggest that GDM *per se* could also be an indicator for Caesarean delivery.

Long-term metabolic comorbidities in mothers: Uncontrolled hyperglycaemia in women with GDM constitutes a status of an increased risk of developing T2DM later in life[145,146]. This had been observed by O'Sullivan and Mahan when they set the first cut-off values for diagnosis of GDM based on the ability of these values to predict later development of T2DM[28,29]. In a recent meta-analysis, Bellamy *et al*[145] demonstrated that women with GDM had 7.43 times the likelihood of developing T2DM as pregnant women without GDM.

Neonatal comorbidities

Neonatal hypoglycaemia occurs as a result of foetal hyperinsulinaemia in response to exposure to high glucose levels from the mother[144]. It can occur even if mothers were not treated during pregnancy as was evident in the ACHOIS[6] and Landon *et al*[7] trials where insignificant difference between the occurrence of neonatal hypoglycaemia in women treated with insulin and in untreated women was reported. In the HAPO study neonatal hypoglycaemia occurred in 2.1% of the total participants and was associated with increased post OGTT maternal glucose but not with fasting glucose levels[4].

Hyperbilirubinemia is likely to be related with increased foetal red cell mass stimulated by decreased oxygen consumption as a result of maternal hyperglycaemia and subsequent foetal hyperinsulinaemia[144]. Hyperbilirubinemia occurred in 8.3% of the HAPO's population but relatively less associated with maternal OGTT glucose levels[4].

Macrosomia, was hypothetically described by Pedersen almost half a century ago as a consequence of foetal, hyperinsulinaemia in response to high transplacental flow of glucose from the mother[3]. This assumption was clearly demonstrated in the HAPO study where increasing maternal glucose level was associated with increased umbilical C-peptide and infant's body weight at birth[4].

Neonatal hypocalcaemia has been reported in

newborn of pregnant women with preexisting diabetes and is likely to be related to hypomagnesaemia[147]. However, its occurrence in neonates born by women with GDM remains infrequent and of little clinical importance[148]. The cause of hypocalcaemia in women with GDM may be related to low vitamin D status in mothers[27].

Respiratory distress syndrome may be the consequence of foetal hyperinsulinaemia interfering with the effect of cortisol on surfactant synthesis[149]. One study showed no difference in the incidence of respiratory distress syndrome in newborns of women with GDM and those born to normoglycaemic women[150].

Other neonatal comorbidities associated with GDM, but to a lesser degree than preexisting diabetes, include hypertrophic cardiomyopathy[151] and major congenital malformations[152].

Long-term metabolic comorbidities in the offspring: The Pedersen hypothesis forms the basis for the current understanding of the effects of intrauterine hyperglycemia on foetal β-cell hypertrophy and adipose tissue and late development of obesity and T2DM in the offspring[3,146]. Children born by women with GDM had an eight times increased risk of developing diabetes or prediabetes when they are 19-27 years old compared with children born from women without GDM[146].

PREVENTION OF T2DM IN WOMEN WITH PRIOR HISTORY OF GDM

Identifying women with GDM at high risk of progressing to T2DM is a key element of early prevention plan. The FPG levels during pregnancy were found to be an important predictor of early postpartum conversion to diabetes[153], and also, as shown by Kim *et al*[154] as the most common factor linked to greater risk of progression to T2DM in the long-term. Area under the OGTT curve, as well as, the degree of glucose level post OGTT, and earlier diagnosis of GDM were also found to be well associated with early postpartum conversion to diabetes[153,154].

The Diabetes Prevention Program (DPP) study showed that intensive lifestyle modification and metformin at a dose of 850 mg twice daily, reduced the incidence of T2DM by 58% and 31% respectively[155]. This beneficial effect has been further confirmed in the long-term follow-up of the original DPP study: DPP Outcomes Study (DPPOS)[156]. In the Troglitazone in Prevention of Diabetes (TRIPOD) study, administration of troglitazone was found to reduce the incidence of T2DM by more than 50% among high-risk Hispanic women with prior GDM. Following withdrawal of troglitazone from the market, the intervention arm was stopped, but the effect of protection from diabetes had persisted for further eight months[157]. In addition, Hispanic women with prior GDM who had finished the TRIPOD study were asked to take part in the Pioglitazone in Prevention of Diabetes (PIPOD) study. After three years, pioglitazone was shown to reduce the incidence of T2DM at a rate of 4.3%/year compared with the rate of 12.1%/year of the original TRIPOD control group[158].

PREVENTION OF GDM

Obese women have a greater risk of GDM than women with normal body weight[17] and it seems logical that behavioral intervention in the form of dietary modification and exercise may result in either reduced risk for GDM or at least halt obesity related gestational comorbidities. However, the current state of evidence does not support this assumption and results were conflicting. For instance, in the United Kingdom Pregnancies Better Eating and Activity Trial (UPBEAT), Poston *et al*[159] did not found behavioral intervention (combination of both healthy diet and physical activity) reducing GDM incidence in obese pregnant women compared with standard antenatal care. Similarly, in the pilot study of Vitamin D And Lifestyle Intervention for GDM prevention (DALI), Simmons *et al*[160] demonstrated a 33% reduction in GDM incidence among obese pregnant women on healthy diet compared with physical activity group. However, this reduction was not significantly different between the two groups[160]. By contrast, in the more recent Finnish Gestational Diabetes Prevention Study (RADIEL), Koivusalo *et al*[161] showed that combined physical activity and dietary modification in obese pregnant women, reduced the incidence of GDM by 39%. The conflicting results obtained could be related to recruitment to RADIEL study of obese women who were identified as high risk on the basis of BMI $\geqslant$ 30 kg/m^2 and/or presence of history of GDM. Previous RCTs on T2DM prevention have shown that the effect of lifestyle intervention was more pronounced in the high risk groups[155]. Another explanation of the results obtained in this study is that, unlike UPBEAT, all individuals recruited were only white women. Moreover, women with history of GDM comprise one-third of total population recruited in the RADIEL study, whereas they form only less than one-tenth in the DALI or UPBEAT studies. This fact means that more women with possible β-cell dysfunction rather than insulin resistance were participated in the RADIEL study[162].

ANTENATAL MANAGEMENT OF GDM

Benefits of treatment

Identification of women with GDM is of utmost importance in order to be engaged in a management plan aiming to reduce both foetal and maternal comorbidities. The ACHOIS[6] and Landon *et al*[7] trials were large RCTs designed to assess benefits of treating GDM. Both studies showed that treatment of GDM reduced the risk of adverse complications including macrosomia, LGA, shoulder dystocia and hypertensive disorders. In a systematic review of 3157 women from seven studies including the ACHOIS and Landon,

the benefit of treatment was assessed using lifestyle and if necessary insulin interventions. An overall significant reduction in macrosomia, LGA, shoulder dystocia, preeclampsia and hypertensive disorders was demonstrated. The risk for perinatal mortality, admission to NICU and birth trauma were also reduced but not statistically significant[163].

In a recent meta-analysis of five RCTs and six retrospective studies the treatment of GDM was found to significantly reduce the risk of preeclampsia, macrosomia and shoulder dystocia but did not present any significant reduction in other comorbidities[164].

Components of antenatal management

Management of women with GDM during antenatal period should consist of medical nutrition therapy (MNT) and weight management, exercise, self-monitoring of blood glucose (SMBG) and pharmacological therapy if required. This should be followed by management during labor and post natal period[144].

MNT and weight management

Women with GDM should be counseled by a dietitian once diagnosis is made to initiate MNT which is the mainstay of any management plan. The aim is to attain normal glycaemic control without ketosis and foetal compromise along with maintenance of adequate weight gain based on prenatal BMI[144]. In determining an appropriate dietary intake for women with GDM, several studies were conducted to compare the different types of diet. Limited caloric intake had been widely recommended for obese women with GDM. This approach was shown detrimental in a randomized prospective study wherein a reduced total caloric intake from 2400 kcal/d to 1200 kcal/d resulted in a significant ketosis among obese women with GDM compared with controls[165]. On the other hand, a caloric intake > 25 kcal/kg per day would prevent both ketosis and foetal growth compromise in obese women[166].

For this reason the ACOG recommends a reduction of the caloric intake to approximately 24 kcal/kg per day for pregnant women with > 120% of the normal body weight[167]. The caloric requirements, composition and distribution throughout the day were also defined by the ACOG (Table 5)[167]. Monitoring weight changes is important to ensure adequacy of dietary therapy and to maintain a weight gain within the recommended rates. The guidelines of the institute of medicine (IOM) in America regarding weight gain were released to assist in prevention of adverse pregnancy outcomes. The IOM recommended a weight gain during pregnancy of 12.5-18 kg for underweight (BMI < 19.8 kg/m^2), 11.5-16 kg for healthy women (BMI 19.8-26.0 kg/m^2), 7-11.5 kg for overweight (BMI 26.0-29.0 kg/m^2), and at least 7 kg for obese (BMI $\geqslant$ 29.0 kg/m^2)[168].

In Langford *et al*[169] study, overweight women who gained weight within the IOM recommendations had a reduced risk for preeclampsia, Cesarean delivery, and macrosomia compared with controls. Whereas those who gained weight over the IOM recommendations had significantly increased risk for preterm birth, macrosomia, and Cesarean delivery[170].

Role of exercise

Exercise is associated with improved insulin sensitivity which might improve both fasting and postprandial glucose levels avoiding the use of insulin in some women with GDM[171]. Exercise has been shown reduce the need of insulin in women with GDM compared with controls[172]. Using data from the Norwegian Mother and Child Cohort (MoBa) study, Magnus *et al*[173] found that exercise reduces the risk of preeclampsia in pregnant women. Likewise, a case-control study showed that in women who underwent regular physical activity, the risk reduction of preeclampsia was 35% compared with controls[174]. The ADA recommends moderate physical activity as part of any management plan, provided clearance from medical or obstetrical problem[175].

SMBG

After the diagnosis of GDM physical activity and MNT is recommended. Additionally frequent SMBG is required to monitor the glycaemic control of the pregnant woman and to determine whether it is adequately achieved or there is need of initiating a pharmacological therapy[10]. It has been reported that frequent SMBG is associated with reduced risk of adverse outcomes[176]. Frequent SMBG based on postprandial rather than preprandial monitoring, has shown to be superior in improving glycaemic control in insulin treated women[177]. Continuous glucose monitoring (CGM) is a novel technology allowing a 24-h assessment of glucose levels. A recent prospective study among Chinese women with GDM has shown a significant improvement of the glycaemic control and a decreased risk of adverse outcomes with the use of CGM technology compared with controls[178].

Pharmacological treatment

Women with GDM who fail to maintain glycaemic targets with nutritional therapy, should initiate pharmacological treatment. In most cases human insulin is the first choice, while some insulin analogues, and certain oral agents may also be used[179]. Although insulin is usually indicated when glycaemic targets are exceeded, some reports from randomized trials suggest initiating insulin based only on foetal ultrasonic parameters, such as increased foetal abdominal girth[180,181]. Recently, Balsells *et al*[182] reported in a meta-analysis that ultrasound-guided management could result in a significant reduction of LGA and foetal macrosomia. In addition, it has been reported that ultrasound-guided management reduces the need for insulin treatment when foetal growth is normal, limiting also the risk of SGA[181].

Insulin

Certain types of insulin are used to treat diabetes in pregnancy. The dose and regimen used is determined based on the severity of hyperglycaemia. Women who

Table 5 The ACOG recommendations of the caloric requirements, composition and distribution throughout the day in pregnant women with diabetes[167]

Caloric requirements		
	Normal BMI	30-35 kcal/kg per day
	< 90% of Normal BMI	30-40 kcal/kg per day
	> 120% of Normal BMI	24 kcal/kg per day
Caloric composition		
	Complex, high-fiber CHO	40%-50%
	Proteins	20%
	Unsaturated fats	30%-40%
Caloric distribution		
	Breakfast	10%-20%
	Lunch	20%-30%
	Dinner	30%-40%
	Snacks	Up to 30%

BMI: Body mass index; CHO: Carbohydrates.

have fasting hyperglycaemia may require a single nocturnal injection of NPH-insulin started at a dose 0.2 units/kg initially, while other women may require only prandial insulin to control post-meal glucose elevations. If both fasting- and post-meal glucose levels are elevated, then a regimen consisting of NPH-insulin twice daily along with short-acting or rapid-acting insulin analogue administered just prior to meals can maintain euglycaemia. In such case, the total daily dose is usually 0.7-1.0 units/kg, divided equally between NPH-insulin and prandial-insulin[179]. Insulin doses are adjusted to attain glycaemic targets and to avoid the risk of hypoglycaemia. Types of insulin that can be used during pregnancy include human insulin both short-acting and NPH-insulin and rapid-acting analogues (lispro and aspart). Long-acting insulin analogues are not extensively studied for use during pregnancy[179,183]. However, a recent RCT showed that insulin detemir was not inferior to NPH-insulin in safety and efficacy[184].

Oral agents

Systematic reviews and meta-analysis of several studies testing oral agents or insulin treatment in GDM have shown that both strategies present comparable safety and efficacy[185-187]. However, the long-term safety of using oral agents in GDM remains obscure[180,184].

Metformin has been shown similar to insulin results in achieving satisfactory glucose control, with no difference in perinatal outcome[187]. When used alone, metformin was found to be associated with less maternal weight gain but with more risk of preterm birth compared with insulin treatment. Furthermore, compared with glyburide, metformin treatment was associated with less macrosomia and less maternal weight gain[186]. In one RCT, Niromanesh *et al*[188] demonstrated a significant reduction in maternal weight gain in women treated with metformin compared with women who received insulin ($P < 0.001$). In addition, a lower rate of birth weight was reported in the neonates born to women in metformin arm compared with women in insulin therapy, but the difference was not significant. In a meta-analysis of six studies involved 1420 women with GDM, Su *et al*[189] showed that treatment with metformin did not significantly increased the rate of neonatal and maternal comorbidities while it was associated with reduced weight gain and neonatal hypoglycaemia. In the same study, however, the use of metformin was associated with an increased risk of preterm birth[189].

Glyburide has also shown similar efficacy and outcomes with insulin[190,191]. However, a recent meta-analysis of 15 studies, demonstrated an increased risk of macrosomia and neonatal hypoglycaemia in women treated with glyburide compared to insulin[186]. Similarly, a recent retrospective study involved 110879 women with GDM, showed that neonatal comorbidities were more frequently encountered when women were treated with glyburide compared with women who received insulin[192]. In one RCT, Casey *et al*[193] demonstrated a significant reduction in fasting glycaemia of women with mild GDM who were treated with glyburide and nutritional therapy compared with placebo. It was also demonstrated that glyburide therapy in combination with diet did not show any benefit in reducing birth weight or improving maternal or neonatal comorbidities[193].

Recommendations of prominent professional bodies

The current ADA (2017)[194] and NICE (2015)[14] guidelines recommend that once GDM is diagnosed, treatment should start with MNT, exercise, weight gain monitoring and frequent SMBG. The glycaemic targets should be maintained at an FPG level of 95 mg/dL, 1-h postprandial of 140 mg/dL and 2-h postprandial of 120 mg/dL. If these targets are not achieved, a pharmacological intervention should be initiated. ADA recommends insulin treatment as the first-line pharmacologic therapy, while NICE suggests metformin in women with GDM who are not achieving glycaemic targets, provided that it is well tolerated and not contraindicated[14,194]. Although there is no clear guidance for their use, ADA recommends to consider both metformin and glyburide for the treatment of GDM, with concerns related to long-term safety as both medicines are crossing the placenta[194]. The NICE guidelines recommend insulin initiation as add-on therapy to the ongoing metformin and lifestyle measures if glycaemic targets were not met. However, insulin may be initiated with or without metformin and lifestyle; if an FPG $\geqslant$ 126 mg/dL, or an FPG 108-124 mg/dL is measured along with the presence of macrosomia or hydramnios. In addition, NICE suggests to consider glyburide in women who failed to achieve glycaemic targets with metformin and refusing to take insulin or in women intolerant to metformin[14]. ADA and NICE do not report any restriction on the type of insulin for use during pregnancy, while NICE prefer the use of

NPH-insulin as the first choice for long-acting insulin and aspart or lispro for rapid-acting insulin analogues[14,194].

MANAGEMENT DURING LABOR AND POSTPARTUM

There is no general agreement on the timing and mode of delivery in women with GDM. However, induction of labor is beneficial in terms of avoiding late perinatal death and obstetric complications related to foetal overgrowth[195]. The ACOG recommend considering elective Caesarean delivery if the estimated foetal weight more than 4.5 kg to prevent birth trauma[167]. Insulin requirement during labor is generally decreased because of increased physical work and also because women may remain fasting for long time. Some women may also need glucose infusion to prevent ketosis[196]. Women who require pharmacological therapy during antenatal period may need insulin during labor to control hyperglycaemia and to check their blood glucose 2-hourly[144]. The Endocrine Society recommends maintaining glucose level in the range of 72-126 mg/dL during labor[197].

Following delivery, most women return back to their previous pre-gestational glycaemic levels soon afterwards. However, some women may continue with hyperglycaemia possibly representing the category of undiagnosed T2DM which is usually present early in pregnancy[144]. For this reason the Endocrine Society recommends to keep checking for glucose level until 72 h following delivery to rule out continuing hyperglycaemia. Treatment is then justified on individual basis if T2DM is diagnosed or, if not, it is recommended to perform a 2-h 75 g OGTT 6-12 wk following delivery to test for glucose intolerance or T2DM[197].

Breast feeding improves weight and glucose tolerance and should be encouraged[198]. Women should also be counseled for the method of contraception and the choices which include hormonal therapy or copper-releasing intrauterine device are acceptable. Hormonal therapy does not appear to increase the risk of developing diabetes[199].

CONCLUSION

To conclude, in the short-term, several maternal and foetal comorbidities were found to be associated with GDM. In the long-term GDM carries a major risk of developing T2DM later in life for both the mother and the offspring. Therefore GDM could be considered as an important health issue. However, a matter of controversy surrounding both the screening and the management of GDM continues to emerge. This may necessitates the need for further studies to demonstrate the benefits of a universal screening and the effects of treatment in reducing the risk of long- and short-term complications. The studies conducted to evaluate the risk factors associated with GDM were observational, thus, and difficult to conclude about possible causality. This was also the case when reviewing the etiology and pathophysiology of GDM, therefore, large prospective clinical trials are needed to extensively study the risk and etiological factors of GDM. Heritability is another important factor influencing the development of GDM, that has not yet clearly elucidated. The conclusions drawn from most of the trials conducted are limited mainly due to the lack of statistical power and the controversial results obtained. Well-designed trials looking for specific genes linkage, expression and association along with functional studies might be needed in the future. Identifying abnormal genes would help in a better understanding of the pathophysiology of GDM and in developing the "keys" for intervention and prevention. Moreover, women with GDM have increased risk of developing T2DM, metabolic syndrome and CVD and therefore identification of women with GDM may trigger the onset of early prevention strategies. There is no universal consensus on how to monitor and treat GDM in terms of caloric control, glycaemic targets and specific pharmacological interventions. However, it is unquestioned that further well-designed RCTs are required in the future to ascertain optimal glycemic targets, and appropriate monitoring of women to assess their risk status for later development of T2DM and CVD.

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